# Ultrastructure of Sperm and Sperm-Egg Interaction in Aculifera: Implications for Molluscan Phylogeny

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## ABSTRACT

All Aculifera, other than neomenioids, fertilize externally with ectaquasperm but these vary greatly in structure between Polyplacophora and Aplacophora, reflecting different modes of fertilization. These structural differences provide us with an additional set of relatively conservative characters with which to examine the phylogeny of Aculifera in relation to other Mollusca and to Bilateria as a whole. Primitive chitons, perhaps similar to *Leptochiton asellus*, probably had ectaquasperm with a large acrosome that penetrated a smooth thick-hulled egg by the release of digestive enzymes and the extrusion of a perforatorium (as is typical of other molluscs). In the majority of extant chitons, such as *Cryptochiton stelleri*, the acrosome has been reduced to a tiny vesicle that digests a pore in the egg envelopes, through which a needle-like portion of the nucleus (replacing the perforatorium), penetrates to fuse with an egg microvillus. The egg hulls of these chitons are elaborated into complex spines or cupules, which may be open or closed. In closed cupule species sperm penetrate between cupules, whereas in open cupule species sperm are attracted inside the cupules to fertilize. Ectaquasperm of Chaetodermomorpha are highly unusual with many derived features indicating a long divergence of the group. Sperm exposed to egg water exhibited an elongation of the apical tube, which suggests a novel polymerization process since a typical subacrosomal granule is absent. Introsperm of Neomeniomorpha are similar to those of internally fertilizing prosobranch snails as well as some polychaete annelids, but are closest in morphology to the parasperm of *Protodrilus* sp., a primitive annelid. This provides additional support for the theory that molluscs and annelids are sister taxa and also suggests that internal, not external, fertilization, may be plesiomorphic to both groups. This would require secondary evolution of ectaquasperm, an idea that has also been suggested for teleost fishes to explain the occurrence of introsperm in basal groups. Furthermore, all extant Platyhelminthes have introsperm but only *Nemertoderma* sp., considered a basal member of the group, has a plausible acrosome, filiform nucleus and midpiece, and typical 9+2 flagellum. Platyhelminthes are widely regarded as basal to the Bilateria which suggests, in contradiction to the widely held belief that early bilaterians fertilized externally, that the common bilaterian ancestor of Platyhelminthes and Eubilateria fertilized internally with introsperm. This indicates that Bilateria are a monophyletic group, their basal members being united by the shared synapomorphies of similar introsperm and internal fertilization, which are absent in all extant Radiata.

## RÉSUMÉ

**Ultrastructure des spermatozoïdes et des interactions spermatozoïdes-oeuf chez les Aculifera: implications pour la phylogénie des Mollusques**

Tous les Aculifera autres que les Neomenioidae ont une fécondation externe avec ectaquaspermatozoïdes dont la structure varie beaucoup entre les Polyplacophora et les Aplacophora, reflétant des modes différents de fécondation. Ces différences structurelles nous fournissent un ensemble supplémentaire de caractères relativement conservés avec lesquels on peut examiner la phylogénie des Aculifera en relation avec les autres Mollusques et l'ensemble des Bilateria. Les chitons primitifs, peut-être similaires à *Leptochiton asellus*, avaient probablement un ectaquaspermatozoïde avec un grand

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acrosome qui pénétrait un oeuf à coque lisse et épaisse grâce à la libération d'enzymes digestives et l'extrusion d'un perforatorium (situation typique des autres Mollusques). Dans la majorité des chitons, tels que *Cryptochiton stelleri*, l'acrosome a été réduit à une petite vésicule qui digère un pore dans les enveloppes de l'oeuf, à travers lequel une portion en aiguille du noyau (qui remplace le perforatorium) pénètre pour fusionner avec une microvillosité de l'oeuf. La coque de l'oeuf de ces chitons est compliquée par des épines ou des cupules, qui peuvent être ouvertes ou fermées. Dans les espèces à cupules fermées les spermatozoïdes pénètrent entre les cupules, alors que dans les espèces à cupules ouvertes les spermatozoïdes sont attirés à l'intérieur des cupules pour la fécondation. Les ectaquaspermatozoïdes des Chaetodermomorpha sont très différents, avec de nombreux caractères dérivés indiquant une longue divergence du groupe. Les spermatozoïdes exposés aux sécrétions des oeufs montrent une élongation du tube apical, qui suggère une polymérisation d'un nouveau type puisque le granule subacrosomien est absent. Les introspermatozoïdes des Neomeniomorpha sont similaires à ceux des Mollusques Prosobranches à fécondation interne et à certaines Annélides Polychètes, mais ont une morphologie très proche du paraspermatozoïde de *Protodrilus* sp., une Annélide primitive. Ceci fournit des arguments additionnels à la théorie selon laquelle les Mollusques et les Annélides sont des groupes-frères et suggère aussi que la fécondation interne, et non externe, pourrait être plésiomorphe dans ces deux groupes. Ceci demanderait une évolution secondaire de l'ectaquaspermatozoïde, une idée qui a déjà été suggérée chez les Poissons Téléostéens pour expliquer la présence d'introspermatozoïdes dans les groupes basaux. De plus, tous les Plathelminthes ont des introspermatozoïdes mais seul *Nemertoderma* sp., considéré comme un membre basal du groupe, a un acrosome plausible, un noyau et une pièce intermédiaire filiforme, et un flagelle typique 9+2. Les Plathelminthes sont généralement considérés comme étant à la base des Bilateria ce qui suggère, en contradiction avec la croyance largement répandue que les premiers Bilateria avaient une fécondation externe, que l'ancêtre commun des Plathelminthes et des Eubilateria avait une fécondation interne avec introspermatozoïde. Ceci indique que les Bilateria sont un groupe monophylétique, leurs membres basaux étant unis par des synapomorphies d'un introspermatozoïde similaire et de la fécondation interne, caractères qui sont absents chez tous les Radiata.

Aculifera consist of two classes, Polyplacophora (chitons) and Aplacophora (chaetoderms and neomenioids) [67, 69]. Among Metazoa, the most primitive groups, Porifera and Radiata, fertilize externally with sperm that have the typical shape, round head, four or five spherical mitochondria and a centriolar satellite complex that harnesses the flagellum at the base of the sperm. To avoid prior connotations of "primitive" and "modified" being associated with sperm shape, JAMIESON and ROUSE [47] devised a new system of nomenclature based on the mode of fertilization, in keeping with FRANZÉN's original hypothesis that sperm structure correlates with biology of fertilization [33, 34]. Two key terms emerged, "ectaquasperm" are characteristic of external fertilizers, whereas "introsperm" characterize internal fertilizers. This system provides for intermediate conditions such as "entaquasperm" [60, 61] and "ect-terrasperm" [80], as well as "plesiosperm" for the archetype [45].

Chitons and chaetoderms have external fertilization with ectaquasperm, although sperm structure and mode of fertilization vary greatly between the groups indicating a long divergence. Neomenioids fertilize internally with introsperm [32], which have a similar morphology to sperm of some primitive annelids [53], gastropods, such as Neritimorpha [15] and Caenogastropoda [13, 40], as well as other invertebrates [2, 3, 35]. The eggs of Aplacophora are relatively unspecialized in relation to surface features but in chitons the hull is usually elaborated into a series of complex spines or cupules, which may be closed or open to the external environment [21, 22, 29]. In hermaphroditic and brooding species the cupules tend to be reduced, sometimes to flattened plates [20, 29]. Self-fertilization is predominant in hermaphroditic species but sperm morphology is unchanged presumably because sperm encounter eggs in a sea water medium in the pallial grooves [20].

Aplacophorans are easily separated from the chitons on the basis of synapomorphies for distichous radula, reduced foot, gonads that open into the pericardium, and small posterior mantle cavity [67, 69]. Furthermore, neomenioids are quite distinct from chaetoderms on the basis of apomorphic characters for sperm structure, other aspects of reproductive biology, as well as general morphology [66, 67, 69]. The main problem arises in trying to distinguish between the different families of chitons.

Chiton phylogeny has been difficult to resolve because potentially useful characters such as radula morphology, which have an extensive fossil record, are virtually unchanged in the chitons [28, 31, 78]. Other characters such as girdle morphology and hull spine tip structure, are highly variable and may have arisen independently in several lineages, or may be polymorphic in a single taxon [28, 71]. Furthermore, previous classifications have been constructed as a series of progressive grades or ascending levels of organization rather than in a way to test phylogenetic affinities [28, 79].

Several authors advocate the use of a classification system for generic and higher taxa based on shell valve morphology alone, because these characters can be corroborated with the fossil record [for review, see 78]. However, as EERNISSE [28] pointed out, any phylogenetic analysis would be strengthened by inclusion of independent morphological characters to avoid coincidental resemblance of form (i.e. of shell valves). EERNISSE first revealed the potential advantages of using gill placement and morphology, as well as egg hull morphology, as independent character sets to test the validity of previous phylogenies based on shell valve morphology alone [28]. SIRENKO followed up on this and studied gill placement and egg hull morphology in 117 species of chitons many of them for the first time [71]. This analysis produced not only a re-arrangement of existing families but the addition of several new ones, including two new super-families, Tonicelloidea and Schizochitonoidea. Lepidochitoninae were included as a subfamily within Tonicellidae, in the superfamily Tonicelloidea. Furthermore, all chitons were grouped in three suborders, Lepidopleurina, Chitonina and Acanthochitonina.

This chapter examines sperm structure and sperm-egg interactions in Aculifera. It has been restricted largely to those species for which complete sperm and egg hull data are available. Thus, families for which sperm and egg data were not verifiable by the author have been omitted from the cladistic analysis. These include Ochmazochitonidae, Loricidae, Schizochitonidae, Juvenochitonidae, Schizoplacidae, and Cryptoplacidae (see [71]). In a few instances key species have been included from other studies to ensure a more complete analysis of a family. This analysis of new sperm and egg characters, when combined with existing morphological characters, has enabled a reassessment of phylogenetic relationships among Aculifera and between them and other taxa.

## RESULTS

### *Sperm structure in Polyplacophora*

Chiton sperm can be classified into one of four main types, depending on the presence of a typical acrosome, type of chromatin condensation, number and position of mitochondria, and presence of an axonemal fibrous complex. The appropriate families for each sperm type are listed on the first line, and follow the recent reclassification of chitons by SIRENKO [71], with the exception of Lepidochitonidae [see phylogeny].

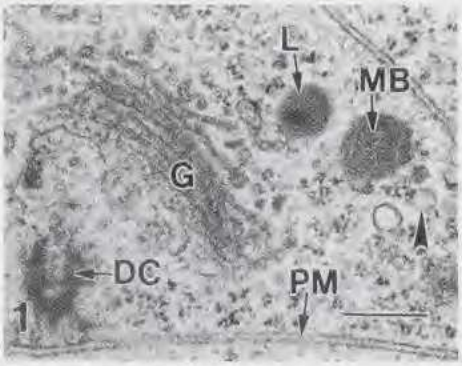
*Sperm type 1*: (Acanthochitonidae, Mopaliidae, Tonicellidae, *Lepidochitona*). This sperm type is probably the most derived as well as the best characterized [16, 17, 19-22, 42, 64, 65]. A detailed description will be given based on this information and following the four arbitrary stages of spermiogenesis described elsewhere [19] and illustrated here in Figure 37. The other sperm types will then be compared to it.

At the onset of spermiogenesis tetrads of Stage A spermatids are interconnected by cytoplasmic bridges (Fig. 4). Chromatin in the spherical nucleus is patchy and granular in appearance. Two centrioles are positioned adjacent to the plasma membrane with the distal one being attached to it via the centriolar satellite complex and annulus (Fig. 1). The proximal centriole is perpendicular to the distal centriole and anterior to it. There are six or more

TABLE 1. — List of species used in the present study, classified according to SIRENKO [71].

| Species studied | Family | Reference |
|---|---|---|
| *Leptochiton asellus* (Linnaeus, 1767) | Lepidopleuridae | [24, 42] |
| *Callochiton castaneus* (Wood, 1875) | Callochitonidae | [24, 42] |
| *Chaetopleura apiculata* (Say, 1834) | Chaetopleuridae | [19] |
| *Acanthopleura granulata* (Gmelin, 1791) | Chitonidae | [4, 24, 71] |
| *Callistoplax crassicostatus* Pilsbry, 1893 | Callistoplacidae | [24, 28, 71] |
| *Stenoplax conspicua* (Pilsbry, 1892) | Ischnochitonidae | [24, 71] |
| *Lepidozona retiporosa* (Carpenter, 1864) | Ischnochitonidae | [24] |
| *Chiton tuberculatus* (Linnaeus, 1758) | Chitonidae | [24, 71] |
| *Onithochiton quercinus* (Gould, 1846) | Chitonidae | [24, 42, 71] |
| *Ischnochiton albus* (Linnaeus, 1767) | Ischnochitonidae | [24, 42, 71] |
| *Lepidochitona dentiens* (Gould, 1846) | Tonicellidae | [19, 28, 29] |
| *Lepidochitona fernaldi* Eernisse, 1986 | Tonicellidae | [19, 20, 28, 29] |
| *Tonicella lineata* (Wood, 1815) | Tonicellidae | [19, 28] |
| *Tonicella marmorea* (Fabricius, 1780) | Tonicellidae | [19, 71] |
| *Nuttalina flexa* (Carpenter, 1864) | Mopaliidae | [24, 28, 71] |
| *Mopalia muscosa* (Gould, 1846) | Mopaliidae | [19, 21] |
| *Mopalia lignosa* (Gould, 1846) | Mopaliidae | [21, 28] |
| *Mopalia ciliata* (Sowerby) | Mopaliidae | [21] |
| *Katharina tunicata* (Wood, 1815) | Mopaliidae | [19, 28, 71] |
| *Acanthochitona viridis* (Pease, 1872) | Acanthochitonidae | [19] |
| *Acanthochitona pygmaea* (Pilsbry, 1893) | Acanthochitonidae | [24] |
| *Cryptochiton stelleri* (Middendorff, 1857) | Acanthochitonidae | [19, 28, 71] |
| *Chaetoderma argenteum* Heath, 1911 | Chaetodermomorpha | [18] |
| *Chaetoderma canadense* Nierstrasz, 1902 | Chaetodermomorpha | [24] |
| *Epimenia australis* (Thiele, 1897) | Neomeniomorpha | [22, 23] |

FIGS 1-9. — Transmission electron micrographs of spermiogenesis in Polyplacophora. **1:** Stage A type 1 spermatid of *Tonicella marmorea* showing Golgi body (G) and associated lysosome (L), multivesicular body (MB) and vesicles (arrowhead). Distal centriole (DC) is attached to plasma membrane (PM) by the annulus. Scale bar = 0.2 µm. **2:** Longitudinal section (L.S.) of stage B type 2 spermatid of *Lepidozona retiporosa* showing condensation of chromatin in 20 nm fine fibres. The proximal centriole (PC) is attached by fibrogranular secretions to the thickened region of nuclear envelope (arrowhead). Scale bar = 1 µm. **3:** L.S. stage C type 2 spermatid of *L. retiporosa*, showing formation of 70 nm coarse fibres. Scale bar = 0.5 µm. **4:** L.S. stage C type 1 spermatid of *Cryptochiton stelleri* showing tight spiralling of fine chromatin fibres. Coarse fibre stage is absent. Note: cytoplasmic bridge joining spermatids (arrowhead); lacuna (L) in nucleus and cross section of flagellum at level of annulus (small arrow). Scale bar = 0.2 µm. **5:** L.S. stage C spermatid of *Tonicella lineata* with fine chromatin fibres twisted anteriolaterally forming extension of nucleus in anterior filament (arrowhead). Two microtubules of manchette surrounding nucleus are visible in superficial section (arrows). Scale bar = 0.5 µm. **6:** Stage D spermatid of *C. stelleri* showing aggregation of nucleopores (P) where nucleus extends into anterior filament. Scale bar = 0.3 µm. **7:** Tip of anterior filament of stage D type 1 spermatid of *Mopalia muscosa* showing acrosome (A) separated from nuclear extension (N) by basal (= subacrosomal) plate (arrowhead). Scale bar = 0.3 µm. **8:** L.S. stage D type 1 spermatid of *M. muscosa* in longitudinal section showing basic form of sperm and position of fibrous complex (FC). Scale bar = 0.2 µm. **Inset**: Close-up of fibrous complex (arrowhead). Scale bar = 0.2 µm. **9:** L.S. stage D type 2 spermatid of *Chaetopleura apiculata* in longitudinal section showing basic form of sperm with large round posterior mitochondria, surrounded by glycogen granules (Gl) on opposite side to basal body (arrowhead). Scale bar = 0.5 µm. Inset: Tip of anterior filament of *L. retiporosa* showing acrosome (A) atop nuclear extension (N). Scale bar = 0.1 µm.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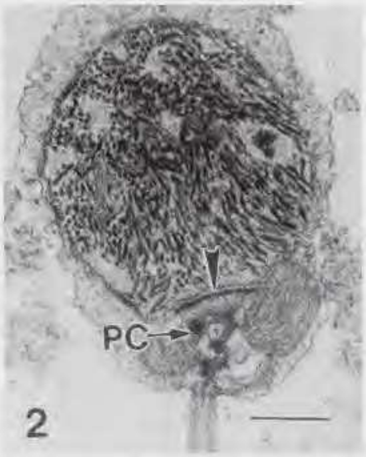
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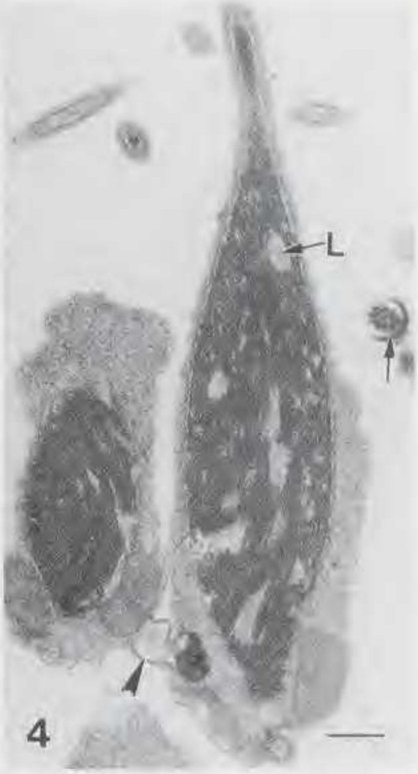
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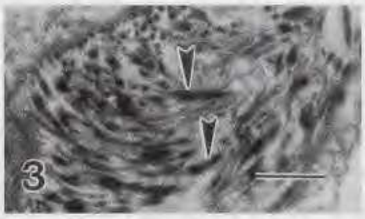
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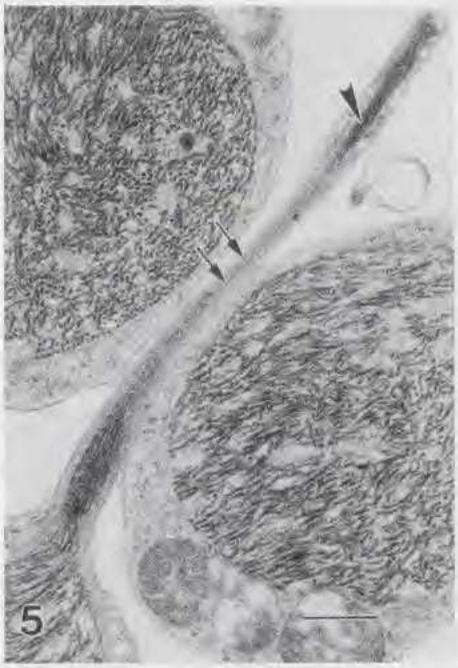
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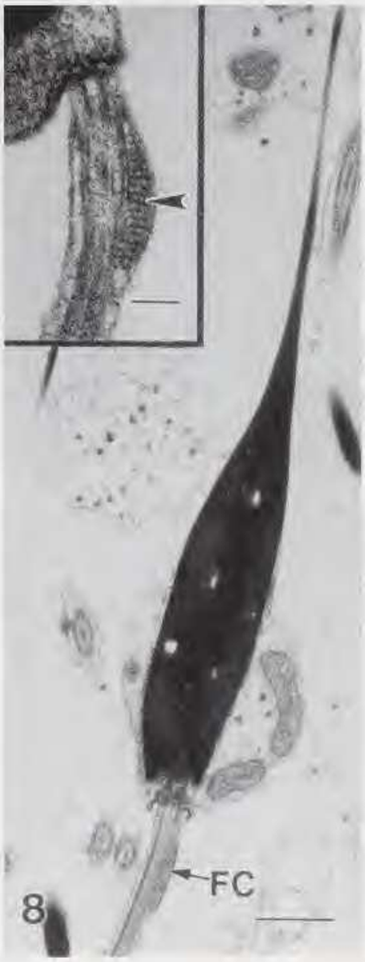
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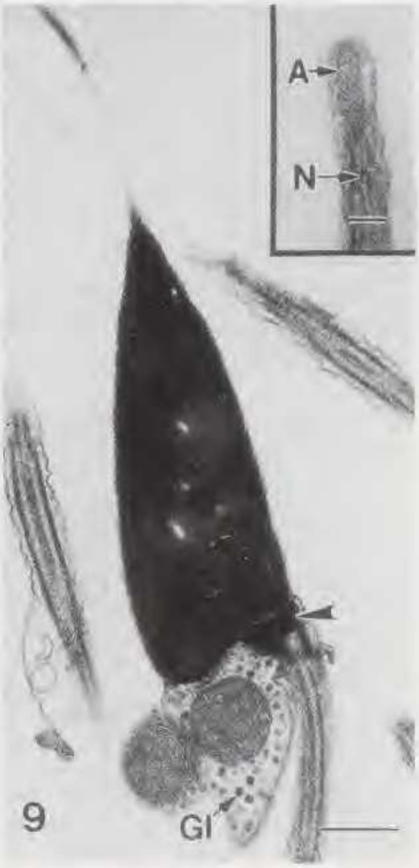
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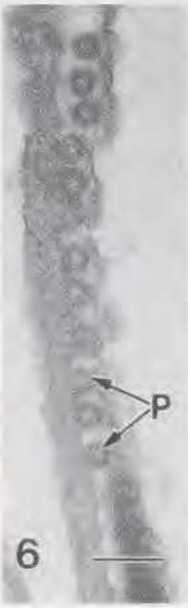
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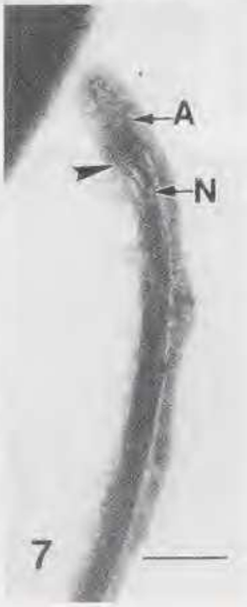
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mitochondria at this stage but as spermiogenesis proceeds they migrate posteriorly and fuse to form between 4 and 7 in the mature sperm. A prominent Golgi body secretes several small proacrosomal vesicles and is usually associated with a lysosome and multivesicular body (Fig. 1). Stage B in all chitons is characterized by the development of the centriolar fossa at the base of the nucleus accompanied by a thickening of the outer nuclear membrane (Fig. 2). The centrioles migrate into this region and the proximal centriole becomes attached by fine fibrous strands to the thickened nuclear membrane (similar to Fig. 2). The flagellum elongates posteriorly. Chromatin condensation progresses forming fine fibres about 20 nm in diameter, which later in this stage become twisted anteriolaterally towards the pointed end of the nucleus (Fig. 5). A microtubular manchette emanates from the centrioles, twists around the nucleus and extends into the pointed tip of the spermatid (Fig. 5). In the Stage C spermatid two granules begin to develop in the acrosome by the fusion of proacrosomal vesicles. The nuclear filament becomes more elongate (Fig. 5) and the nucleopores aggregate at its base (Fig. 6). The rest of the nucleus appears to be devoid of nucleopores at this stage. Glycogen granules are formed in the midpiece, near the Golgi body and mitochondria. The basal body is slightly offset and mitochondria begin to group above it on the same side, and below it on the opposite side. In Stage D the spermatids mature and the microtubular manchette disappears. The acrosome, containing two granules, is separated from the nuclear filament by a basal (= subacrosomal) plate (Fig. 7). The nucleus and nuclear filament become homogeneously dense with occasional lacunae in the main body (Fig 8). The nucleus is bullet-shaped and subtends an offset basal body comprising proximal and distal centrioles interconnected by fibrogranular secretions. Mitochondria are found laterally above the centrioles and basally opposite them. The centriolar satellite complex and annulus are housed in the collar, which is an evagination of plasma membrane that extends posterior to the distal centriole and around the axoneme. A fibrous complex forms adjacent to the axoneme near the annulus, on the same side as the basal body (Fig. 8, inset). The elongate flagellum terminates in a narrow endpiece, which contains only the central microtubules and is similar in length to the nuclear filament. The Golgi body, rough endoplasmic reticulum, lysosome, multivesicular body, as well as excess cytoplasm are eliminated via the cytoplasmic bridge into a central cytoplasmic droplet, which is sloughed off thus freeing the four mature spermatozoa of the cohort.

*Sperm type 2*: (Chaetopleuridae, Chitonidae, Callistoplacidae, Ischnochitonidae). Type 2 chiton sperm has been described previously for 12 species [4, 19, 42, 63-65], and is represented here by *Lepidozona retiporosa*. Type 2 sperm develop similarly to Type 1 with the following exceptions: firstly, following the fine fibre phase (fibres are 20 nm in diameter) of chromatin condensation (Fig. 2), there is a coarse fibre phase (fibres are 70 nm in diameter) (Fig. 3), before the nucleus becomes homogeneously dense (Fig. 9). Secondly, the midpiece has a distinctive

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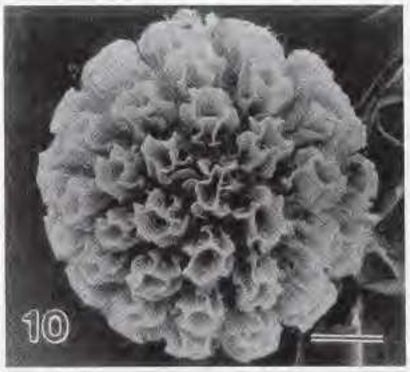
FIGS 10-20. — Sperm-egg interactions in Polyplacophora. **10:** Mature open cupule egg of *Mopalia ciliata*. Scale bar = 50 µm. **11:** Open cupules of polyspermic egg of *M. muscosa*. Scale bar = 20 µm. **12:** Polyspermic egg of *M. muscosa* with cupules removed revealing preponderance of sperm inside cupule profiles. A few sperm penetrate area between cupules. Scale bar = 10 µm. **13:** Mature closed cupule egg of *Lepidochitona dentiens*. Scale bar = 50 µm. **14:** Mature spinous egg of *Stenoplax conspicua*. Scale bar = 50 µm. **15:** Polyspermic eggs of *S. conspicua* with sperm between spines. Bifurcations of spines now are closed, but were spread apart when spawned. Scale bar = 10 µm. **16:** Polyspermic egg of *M. muscosa*: sperm penetrating hull inside cupule. Scale bar = 2 µm. **17:** Light micrograph of 1 µm section of sperm of *T. lineata* penetrating hull and vitelline layer to inject nuclear material into egg (arrowhead). Scale bar = 2 µm. (From [16]). **18:** Portion of maturing egg of *Acanthopleura granulata*, showing polymorphic spines. Short bifurcating spines occurring between medium to long single tipped spines with caps. Scale bar = 10 µm. **19:** Polyspermic egg of *S. conspicua* in plan view showing sperm penetrating hull at junctions of cupule bases (arrowheads). Scale bar = 5 µm. **20:** T.E.M. of fertilizing sperm penetrating hull of *S. conspicua*. Characteristic dense outer layer of hull, has been dissolved around penetrating sperm. Scale bar = 2 µm.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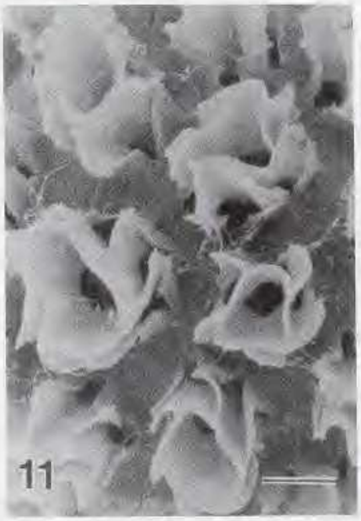
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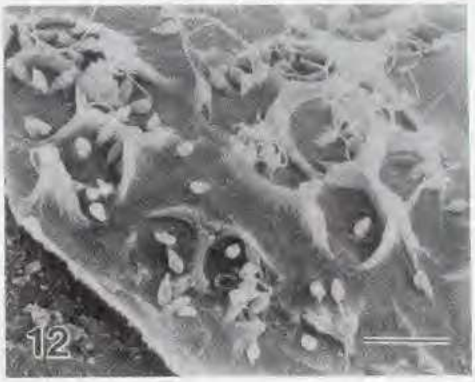
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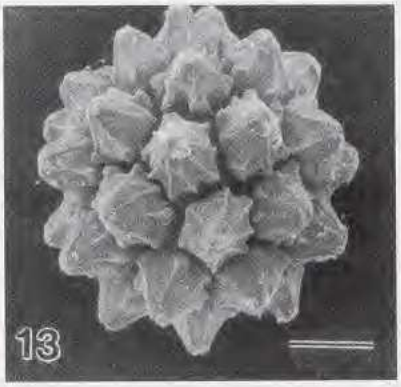
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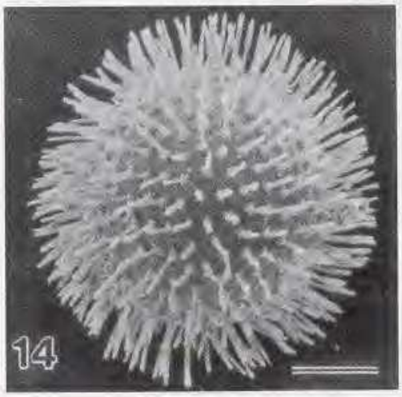
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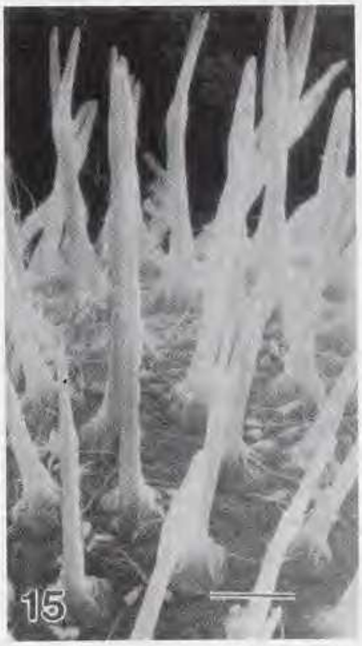
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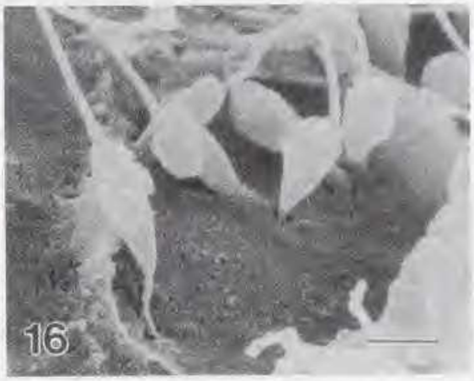
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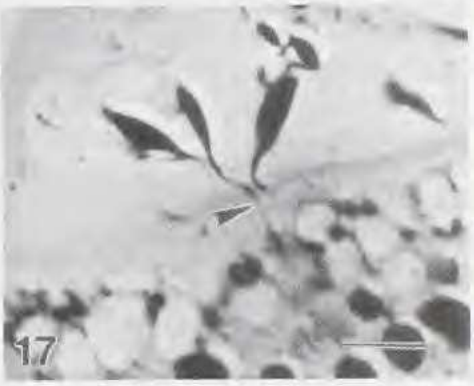
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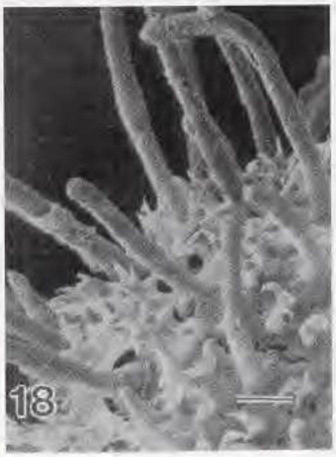
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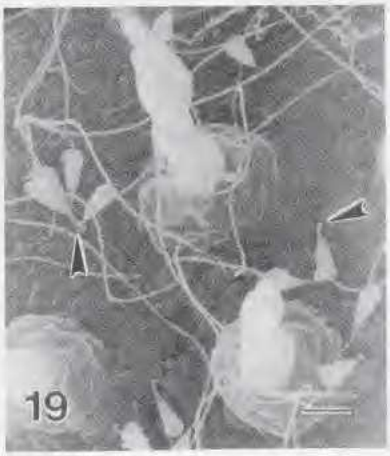
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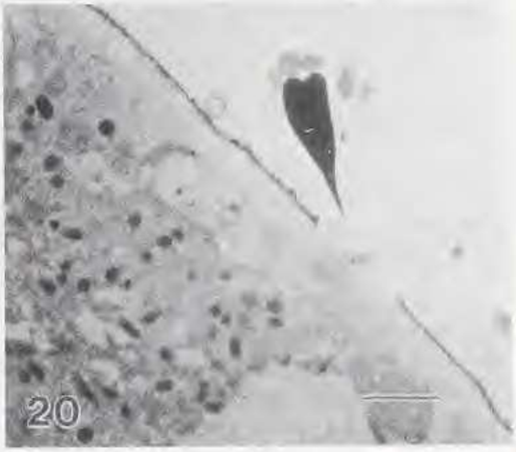
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appearance because of the lack of lateral mitochondria and the position of basal mitochondria (Fig. 9). Frequently two mitochondria are adjacent to the nucleus with a third one below them. This extends the midpiece and collar posteriorly. Thirdly, the basal body is more offset than in Type 1. This and the position of mitochondria gives the Type 2 sperm its characteristic shape (Fig. 9). Fourthly, the fibrous complex is absent or poorly developed, but at least in some species the plasma membrane is thickened at the same site as the fibrous complex of Type 1 sperm. The acrosome is more uniform in appearance, lacking distinct apical and basal granules (Fig. 9, inset).

*Sperm type 3*: (Callochitonidae). The third type of sperm has been described only in Callochitonidae [42, 64], represented here by *Callochiton castaneus*. Sperm of Hanleyidae, a fairly primitive family, have not yet been examined.

Type 3 sperm is characterized by the plesiomorphic condition of a central basal body and flagellum, surrounded by five mitochondria at the base of the nucleus. The mitochondria are slightly derived because two of them are elongate whereas three are spherical [42].There is no fibrous body or unilateral reinforcement of the plasma membrane near the annulus. In other respects the sperm is similar to Type 2 with a similar pattern of chromatin condensation.

*Sperm type 4*: (Lepidopleuridae). This sperm type, considered plesiomorphic to chitons, is known only from the species, *Leptochiton asellus* [42]. The sperm is unique among chitons in having a fairly typical molluscan acrosome. A detailed diagram summarizing spermiogenesis in *L. asellus* is given in Figure 36 (based on [42] and unpublished observations).

In Stage A spermatids a large proacrosomal vesicle (1 µm in diameter) is evident posterior to the nucleus and connected to the plasma membrane by flocculent material, as occurs in aplacophorans and other molluscs. During Stages B and C the nucleus becomes oval and chromatin condensation proceeds from granular to short fine fibres 20 nm in diameter, then to short coarse fibres, 70 nm in diameter. The fibres appear much shorter than in other chiton groups, reflecting variation in the pattern of condensation. The mitochondria fuse to form five spheres surrounding a central basal body and unspecialized flagellum. In Stage C the proacrosomal vesicle, independent of the Golgi body, differentiates into the elongate acrosome cone and subacrosomal granule, which becomes separated from the nucleus by the subacrosomal plate. The subacrosomal granule apparently lacks filaments, as in some gastropods. In Stage D, the nucleus becomes homogeneously dense and bullet-shaped with no anterior filament. The flagellum terminates in a short filamentous endpiece containing only the central microtubules.

### *Sperm-egg interaction in Polyplacophora*

Chiton eggs are surrounded by a vitelline layer as well as a hull, both of which appear to be secreted by the egg [58, 59]. With few exceptions the hulls of chiton eggs are characterized by elaborate projections which vary from spinous to cupular [16, 17, 20-22, 28, 29, 31, 43, 56, 58, 70, 71]. By contrast the hull of *Leptochiton asellus* (Lepidopleuridae) is smooth [42]. In brooding forms, which include several hermaphroditic species, the cupules are often reduced but retain the basic morphology of the group [20, 28, 29]. There are three main types of sperm-egg interaction which correlate with egg hull structure as follows: Type 1. Cupular projections; Type 2. Spinous projections; Type 3. Smooth hull. Variation in the size, shape and structure of hull projections has profound influences on; the site and mechanism of fertilization [16, 17, 20-22], the drag on the egg and therefore its sinking rate [21], the aggregation of eggs in strings and clusters and attachment to substrates [28, 29, review: 56] and therefore the target size for sperm, as well as the limitation of numbers of eggs that can be brooded [20, 28, 29].

*Egg type 1*: Cupular projections. (Lepidochitonidae, Tonicellidae, Mopaliidae, Acanthochitonidae). In cupular species the projections may be open or closed, which directly influences where the sperm penetrate the egg. In species with open cupules, such as in *Mopalia ciliata*, (Fig. 10), sperm preferentially swim inside the cupules, become segregated into one of seven channels and penetrate the hull where it overlies the egg membrane (Figs 12, 16). At this point the hull is thinnest. The hull can be penetrated between cupules but this is rare and has only been observed in polyspermic eggs, created artificially by using a sperm concentrate (Fig. 12). No micropores have been observed in the hull of these species. The acrosome appears to contain two granules, which in *Tonicella lineata* are exhausted sequentially as the sperm penetrates the two egg envelopes, fuses with an egg microvillus and injects the nuclear material into the egg [16, 17] (Fig. 17).

During ontogeny cupules are covered by follicle cells [58, 70], which are thought to be involved in their morphogenesis but not their secretion [58, 59] distinguishing this envelope from the chorion of other animal groups. An immature open cupule egg can easily be mistaken for a closed cupule one. A good example is *Tonicella lineata*, which only becomes obviously open cupule in form when it sheds follicle cells close to spawning [16, 17]. Thus it is very important to make definitive observations only on naturally spawned eggs. Chitons collected during the breeding season [review: 56] may be kept in culture dishes in running sea water, until spawning occurs. Usually this happens one or two days after collection. Males usually spawn first and appear to stimulate females [56]. In species with closed hull cupules, such as *Lepidochitona dentiens* (Fig. 13), sperm must penetrate the egg envelopes between projections. In *L. dentiens*, micropores are present between cupules in the mature egg that provide sperm with direct access to the vitelline layer [20, 21]. Preliminary evidence suggests that sperm acrosomes may be further simplified in this and similar species by having a single granule [21]. In the brooder *L. fernaldi*, the structure of the egg is the same as *L. dentiens* except that cupules are reduced to flattened plates [20].

*Egg type 2*: Spinous Projections. (Hanleyidae, Callochitonidae, Schizochitonidae and Loricidae [71]; Chaetopleuridae, Chitonidae, Ischnochitonidae, Callistoplacidae). In chitons with spinous projections, such as *Stenoplax conspicua* (Fig. 14), the sperm penetrates the hull between spines, frequently at the junction of their bases (Figs 15, 19). Spines assume a wide variety of forms from complex, as in *Chaetopleura apiculata*, to simple as in *Hanleyella asiatica* [71]. Simple spines have a wide variety of tip formations, including one to seven-fold symmetry. Spinous tips may be pointed and bifurcate, as in *S. conspicua* (Fig. 15) or petalloid, as in *Chiton tuberculatus*. However, there appears to be considerable polymorphism within genera [71], making it difficult to use tip form as a character in phylogenetic analysis. The egg hull of *S. conspicua* (and possibly other spinous hulled species), has an outer dense layer in addition to the typical hull. This thin dense layer is digested by the entrance of the sperm, presumably because certain acrosomal enzymes are released (Fig. 20). The area of the hull disrupted by sperm entrance is much greater than the width of the anterior filament and cannot be accounted for just by gaps between spine bases, visible in some micrographs (Fig. 19).

*Egg type 3*: Smooth Hull. (Lepidopleuridae). In *Leptochiton asellus*, the only chiton species known with a smooth-hulled egg, fertilization is presumed to occur anywhere on the egg surface. However, sperm-egg interaction has not been observed in any lepidopleurids. The presence of a typical molluscan acrosome is coincident with an egg hull that is ten times thicker than in other chitons and probably reflects penetration by a perforatorium as is typical of other molluscs and invertebrates [42]. For these and other morphological reasons (see phylogeny section), Lepidopleuridae are considered basal to Polyplacophora.

### *Sperm structure and sperm-egg interaction in Aplacophora*

Class Aplacophora may be divided into two subclasses, the Neomeniomorpha and the Chaetodermomorpha [67, 69]. Neomenioids are considered basal to Aculifera and to Mollusca as a whole, based on recent morphological [67] as well as molecular analyses [62], whereas chaetoderms are considered to be derived, having diverged early from the molluscan lineage [69].

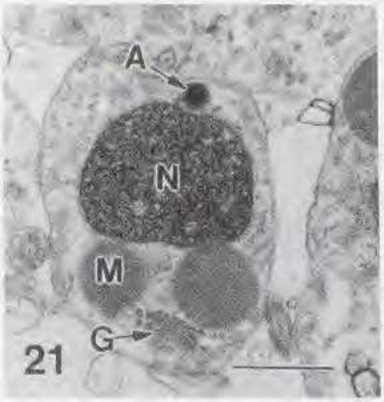
Neomenioids and chaetoderms have radically different modes of reproduction. Neomenioids are monoecious and fertilize internally, with introsperm being stored in seminal receptacles [68, 69]. Chaetoderms are dioecious and fertilize externally, by releasing ectaquasperm into the ambient sea water [18, 69]. These sperm types are quite different from those of chitons.

*Sperm of Neomeniomorpha*: Epimenia australis. Spermiogenesis in *E. australis* is summarized diagrammatically in Fig. 38. Spermiogenesis of the introsperm of *Epimenia* is very similar to that of the parasperm of the primitive annelid, *Protodrilus*, as well as introsperm of some prosobranchs [13, 15, 40]. Stage A is similar to what has been described for chitons. In stage B the sperm still has the plesiosperm form (Fig. 21). During this stage the centrioles migrate to the centriolar fossa dragging the plasma membrane with them and causing it to invaginate where it is attached to the annulus. This creates a temporary flagellar canal. The mitochondria move posteriorly and begin to fuse at the base of the nucleus, initially into four or more spheres. At this time a Golgi body is posterior to the nucleus and mitochondria (Fig. 21). In stage C, chromatin condensation progresses from granular to fine fibres 20 nm in diameter, which become twisted as the nucleus elongates (Fig. 22). During this stage the Golgi body secretes numerous vesicles which contribute to a large mass of dense granular material at the base of the nucleus around the axoneme. The annulus breaks away from the distal centriole and begins migrating posteriorly towards the junction between midpiece and glycogen-piece. The dense granular mass becomes reorganized around the proximal and distal centrioles where it contributes to the formation of the basal body as well as to the adjacent peribasal body. Similar granular secretions are organized into a spiral ridge around the axoneme down to the annulus (Fig. 38C).

The Golgi body plays an active role in forming the spiral ridge much like that observed previously in *Nerita picea* [15]. Subsequently, the Golgi body migrates again to the apex of the sperm adjacent to the acrosome cone, which to this point has been developing in the absence of the Golgi body (Fig. 38C). An extracellular striated cone, coated with fine fibrous strands, begins

FIGS 21-28. — Spermiogenesis in Neomeniomorpha: *Epimenia australis*. **21:** L.S. stage B spermatid showing basic plesiosperm form. Apical acrosome (A), Golgi body (G) and spherical mitochondria (M) at base of nucleus containing granular chromatin (N). Scale bar = 1 µm. **22:** L.S. stage C spermatid showing twisting and spiralling of chromatin fibres in nucleus (N). Acrosome (A) is at tip of spiralled nucleus (out of section). Golgi body (G) is once again associated with the acrosome (or else there 2). Large fused mitochondria (M) are beginning to elongate around axoneme, which has been reinforced by spiral ridge (arrowhead). Scale bar = 1 µm. **23:** Stage D spermatid. L.S. of acrosome (A), subtending sub-acrosomal granule (SG) and separated from dense nucleus by sub-acrosomal plate (arrowhead). Note: striated extracellular cone (EC) on thickened membrane at tip of acrosome. Scale bar = 0.2 µm. **24:** L.S. stage D spermatid showing basal body (BB) in posterior indentation of nucleus, which is blocked by peri-basal body (PB). Note also: mitochondria (M) and sections of spiral ridge (R). Scale bar = 0.2 µm. **25:** Stage D spermatid. L.S. of junctions between mid-piece and glycogen piece showing annulus adjuncts (AnA), mitochondrion (M), spiral ridge (R) and annulus (arrowhead). Scale bar = 0.2 µm. **26:** Stage D spermatid. L.S. glycogen piece showing glycogen granules (arrowheads). Scale bar = 0.3 µm. **27:** Cross Section (C.S.) stage D spermatids at level of acrosome (A), nucleus (N) and mitochondrion (M). Note single break in mitochondrion (arrowhead), which forms longitudinal slit. Scale bar = 0.2 µm. **28:** L.S. end-piece of stage D spermatids, showing density (arrowhead) at termination of central microtubules. Scale bar = 0.2 µm.

FIGS 29-35. — Sperm and eggs of Chaetodermomorpha. **29:** Stage C spermatid of *Chaetoderma canadense* with granular chromatin in nucleus (N), Golgi body (G) is in process of forming apical horn and apical tube in anterior of spermatid. Scale bar = 0.5 µm. **30:** Stage C spermatid of *C. canadense* showing acrosome (A) at tip of apical tube (AT), that caps apical horn (AH). Apical tube is projected over large Golgi-derived vesicle (V). One of five mitochondria (M) lies at base of nucleus. Scale bar = 0.5 µm. **31:** C.S. mid-piece of *C. argenteum* showing five mitochondria encircling centrioles. Scale bar = 0.5 µm. **32:** Light micrograph of mature egg of *C. argenteum* with apparent hull (arrow). Scale bar = 30 µm. **33:** Enlarged area of *C. argenteum* egg showing periodic bumps with short spinous or tubular projections (arrows). Scale bar = 20 µm. **34:** L.S. of acrosome (A) and apical tube (AT) of *C. canadense*. Note attachment of acrosome to plasma membrane (arrowhead). Scale bar = 0.2 µm. **35:** S.E.M. of *C. argenteum* sperm exposed to egg water for ten minutes. Apical tube (AT) of upper sperm is longer than lower sperm. Elongation can be at least 1.5x original length. Note also apical horn (AH), acrosome (A) and mitochondria (M). Scale bar = 2 µm.



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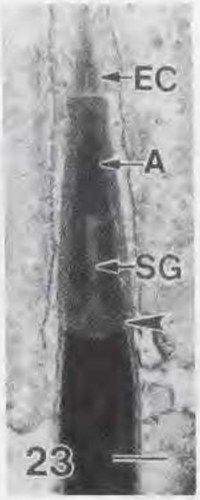

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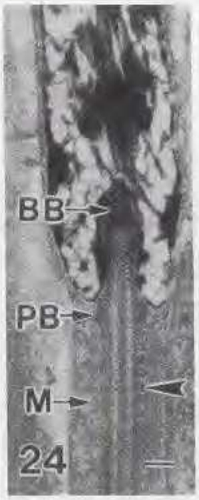

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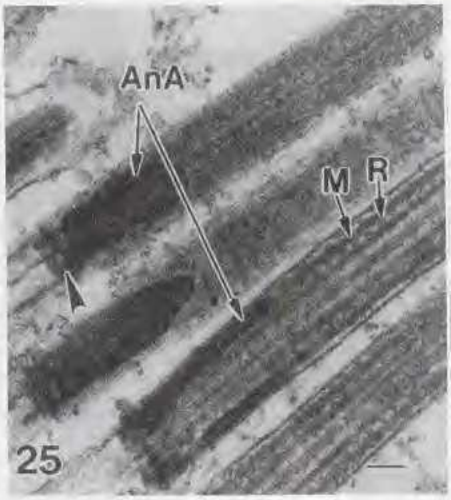

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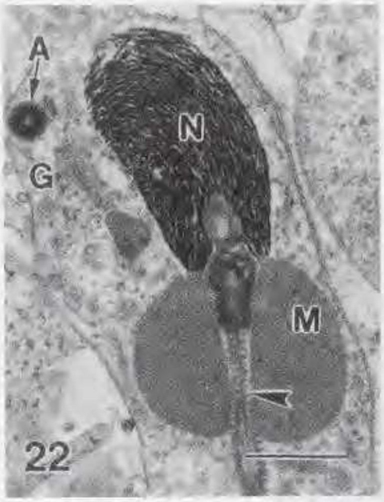

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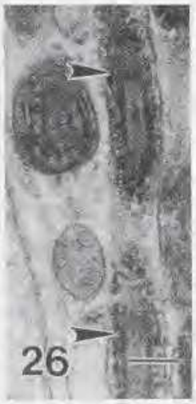

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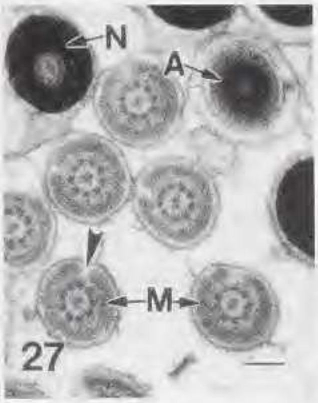

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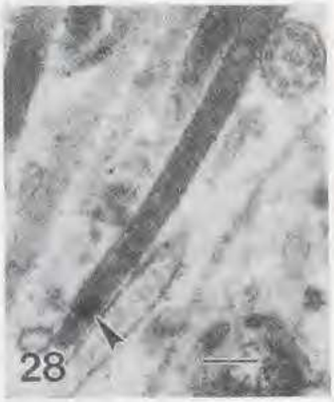

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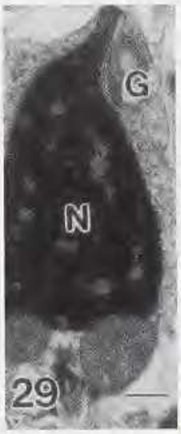

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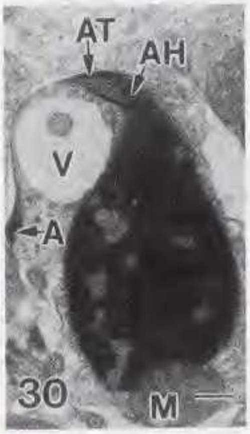

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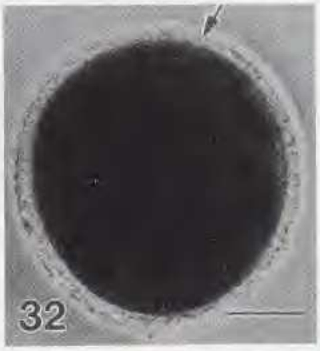

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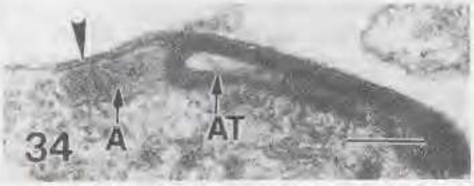

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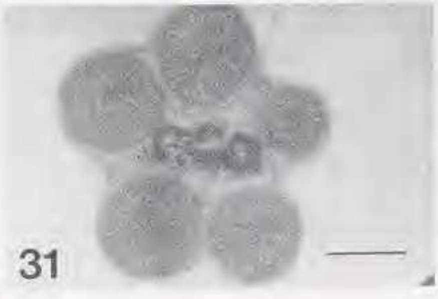

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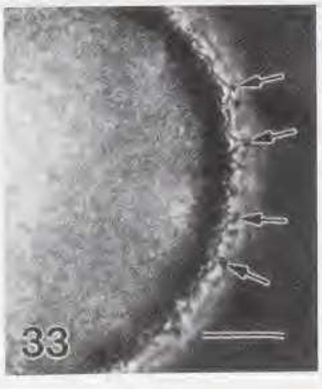

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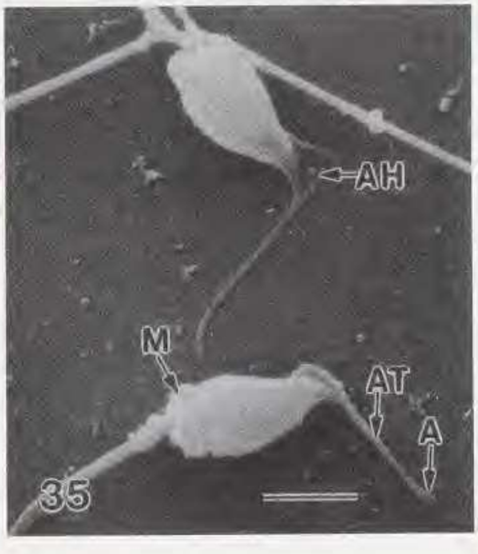

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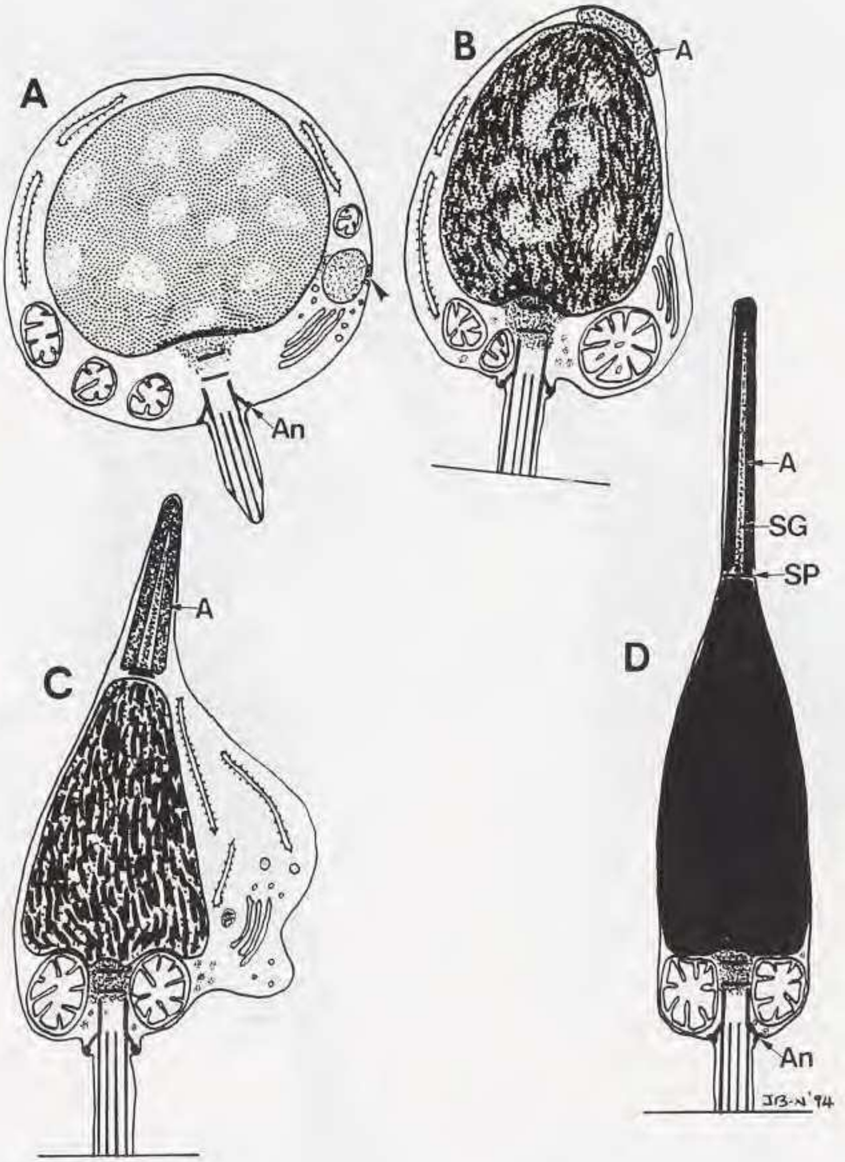
to form near the tip of the acrosome, between the sperm and the somatic (Sertoli) cell. Its function is unknown.

The basal body becomes housed in a posterior indentation of the nucleus, which is closed off by the peribasal body (Fig. 24). The annulus breaks away from its connections to the distal centriole and leads the posterior migration of the mitochondria. The acrosome cone is fully developed and subtends a subacrosomal granule, separated from the dense nucleus by a subacrosomal plate. The two mitochondria begin to elongate and later fuse to form a single Nebenkern enclosing the axoneme but for one longitudinal slit down its length (Fig. 27). In stage D the mitochondrion extends from the base of the nucleus to the tip of the annulus adjuncts, which are two hollow, semicylindrical structures that form above the annulus on either side of the axoneme (Fig. 25). The annulus occupies an invagination of the plasma membrane at the junction between midpiece and glycogen piece (a site typical of many metazoan introsperm) (Fig. 25). The glycogen piece is filled with granules of glycogen (Fig. 26), which tend to be grouped over the nine axonemal doublet microtubules. This aggregation is similar but less ordered than in gastropods. The axoneme tapers towards the endpiece, where it is reduced to just the central microtubules that terminate in a characteristic density (Fig. 28).

*Sperm-egg interaction in Neomeniomorpha*: Sperm-egg interaction in *Epimenia* has not been observed. The outer egg envelope appears to be unspecialized, which suggests that fertilization may occur anywhere on its surface. Fertilized eggs are brooded prior to release as juveniles [68].

*Sperm of Chaetodermomorpha*: Chaetoderma canadense, *and* C. argenteum. See diagrammatic summary of spermiogenesis in Fig. 39. The ectaquasperm of *C. argenteum* [18] and *C. canadense* [22] are highly unusual. Spermiogenesis begins in a similar way to *Leptochiton* but after forming the proacrosomal vesicle the Golgi body migrates to the apex of the nucleus (Fig. 29) where it becomes involved in producing three key structures: the apical horn, the apical (dense) tube and a large vesicle below the tip of the nucleus (Fig. 30). The apical tube grows out over the large vesicle to contact the proacrosome on the plasma membrane (Fig. 30). Glycogen granules are deposited around the apical horn as well as the mitochondria. At this time there are two flagella, proximal and distal, produced by the respective centrioles. The distal flagellum encircles the nucleus and exits posteriorly. The proximal flagellum grows anteriorly through the cytoplasm and exits at the tip of the nucleus, alongside the developing apical tube, perhaps acting as a guide [18]. Mitochondria fuse to form five spheres surrounding the centrioles at the base of the nucleus. At the completion of spermiogenesis the proximal flagellum separates at the centriole and is eliminated anteriorly with the residual cytoplasm (including Golgi body, large vesicle, lysosome, multivesicular body, endoplasmic reticulum, etc.) [18]. Conversely, in most Metazoa the residual cytoplasm is eliminated from the midpiece region. The distal flagellum unwinds and exits posteriorly in the usual position for molluscs. The chromatin condenses from granular, through patchy, to homogeneously dense with a few lacunae in the mature spermatid [18]. The distal centriole is harnessed to the plasma membrane by the annulus via the centriolar satellite complex, which typifies ectaquasperm [74] and is plesiomorphic for introsperm [2, 73, 77]. The axoneme has a typical 9+2 configuration and terminates in just the two central microtubules as in other Aculifera.

The mature sperm is easily mistaken for a chiton sperm because the apical tube resembles the anterior filament (Fig. 35) [32]. However, the apical extension is directed anteriolaterally and is of completely different construction [18]. It contains no nuclear material and comprises two unique structures, the apical horn and apical tube (Figs 30, 35), the functions of which remain unknown. There is, however, little doubt that the acrosome lies at the tip of the apical tube, as this vesicle forms from the Golgi body in much the same way as proacrosomes of some other molluscs, including *Leptochiton*.



FIGS 36-39. Diagrammatic representation of four arbitrary stages (A-D) of spermiogenesis in Aculifera following descriptions given in text.

FIG. 36. — Spermiogenesis in *Leptochiton asellus*. **A**: Pro-acrosome (arrowhead) formed from Golgi vesicles, is migrating to apex of nucleus along plasma membrane. Annulus (An) connects distal centriole to plasma membrane. Nucleus contains granular chromatin and in the cytoplasm are several small mitochondria and rough E. R. **B**: Pro-acrosome (A) is atop nucleus, chromatin is forming short fine fibres in nucleus. Mitochondria are at base of nucleus with Golgi body. **C**: Acrosome (A) is elongating in absence of Golgi body. Chromatin fibres are thicker and fused, enlarged mitochondria surround centrioles. **D**: Fully developed acrosome subtends a subacrosomal granule and is separated from dense nucleus by sub-acrosomal plate. Five uniform spherical mitochondria surround centrioles. Annulus (An) attaches distal centriole to plasma membrane via satellite complex [24, 42].

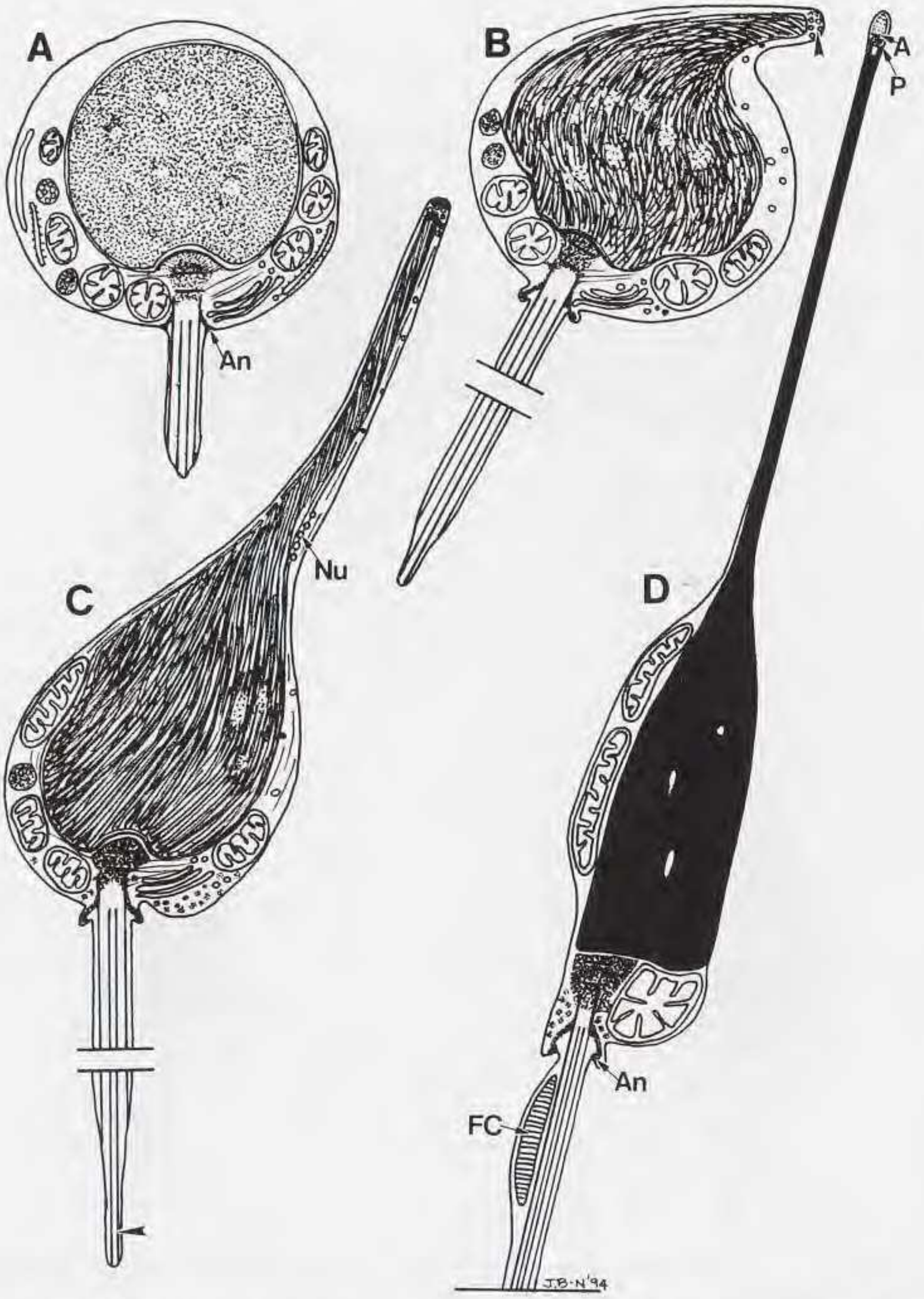


FIG. 37. — Spermiogenesis in *Cryptochiton stelleri*. **A**: Similar to *L. asellus* but no pro-acrosome forms. Individual pro-acrosomal vesicles are visible near Golgi body. **B**: Chromatin condensing in fine fibres is twisting anterio-laterally in nucleus. Proacrosomal vesicles are visible at apex of spermatid (arrowhead). Mitochondria have accumulated at base of nucleus with Golgi body. **C**: Anterior filament is elongating with acrosome forming at its tip. Nucleopores (Nu) have aggregated at base of anterior filament. Tip of tail contains only central microtubules (arrowheads). **D**: Bullet shaped sperm has long needle-like filament with acrosome (A) at its tip. Acrosome is separated from dense nuclear extension by a basal (= subacrosomal) plate (P). Mitochondria are positioned laterally and below nucleus, adjacent to centrioles. Annulus (An) attaches distal centriole to plasma membrane. Fibrous complex (FC), characterizing Type 1 sperm, is visible below annulus.

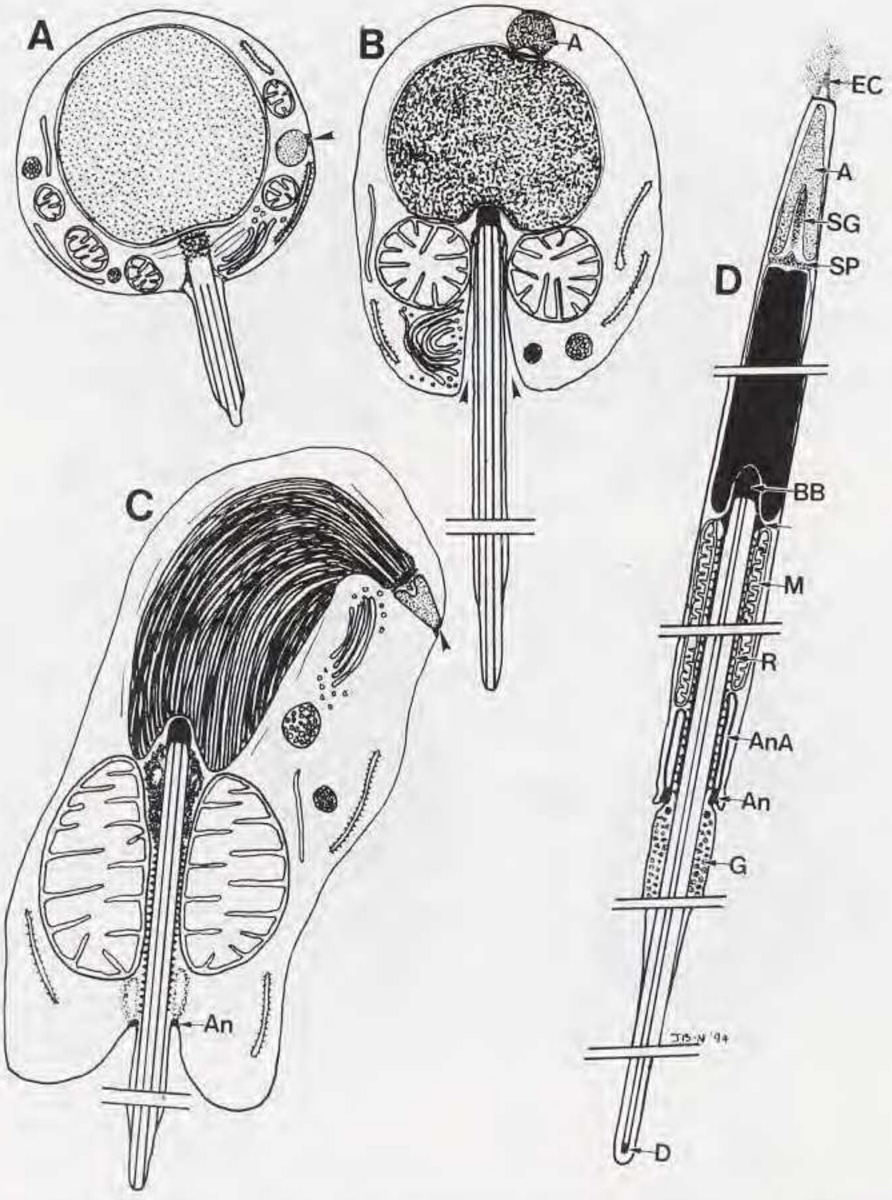


FIG. 38. — Spermiogenesis in *Epimenia australis*. **A**: Plesiosperm stage like that in Figs 36 & 37. Pro-acrosomal granule (arrowhead) is attached to plasma membrane by fibro-granular material. **B**: Pro-acrosome (A) is visible atop nucleus. Chromatin is granular but more tightly packed than in stage A. Mitochondria are fusing and aggregating at base of nucleus. Basal body, comprising fused proximal and distal centrioles, has migrated into centriolar fossa. Large active Golgi body is positioned next to temporary flagellar canal (arrowheads). **C**: Pro-acrosome (arrowhead) is attached to plasma membrane atop nucleus with Golgi body nearby. Chromatin has condensed into elongate fine fibres which are twisted inside nucleus. Annulus has broken away from distal centriole and is migrating posteriorly everting flagellar canal as it does so. Dense granular material surrounds apex of axoneme below basal body and a spiral ridge is evident posterior to this. Two large mitochondria are elongating posteriorly. **D**: Late spermatid with introsperm form. Extracellular striated cone (EC) is atop acrosome (A), which subtends acrosomal granule (SG) and is separated from dense nucleus by sub-acrosomal plate (SP). Basal body (BB) is housed in basal indentation of nucleus, blocked posteriorly by peri-basal body (arrow). Mitochondria (M) form an almost complete sheath around axoneme, reinforced by spiral ridge (R). Annulus (An) is in characteristic junction of mid-piece and glycogen piece and extends anteriorly as annulus adjuncts (AnA). Note also: Glycogen (G) and terminal density (D).

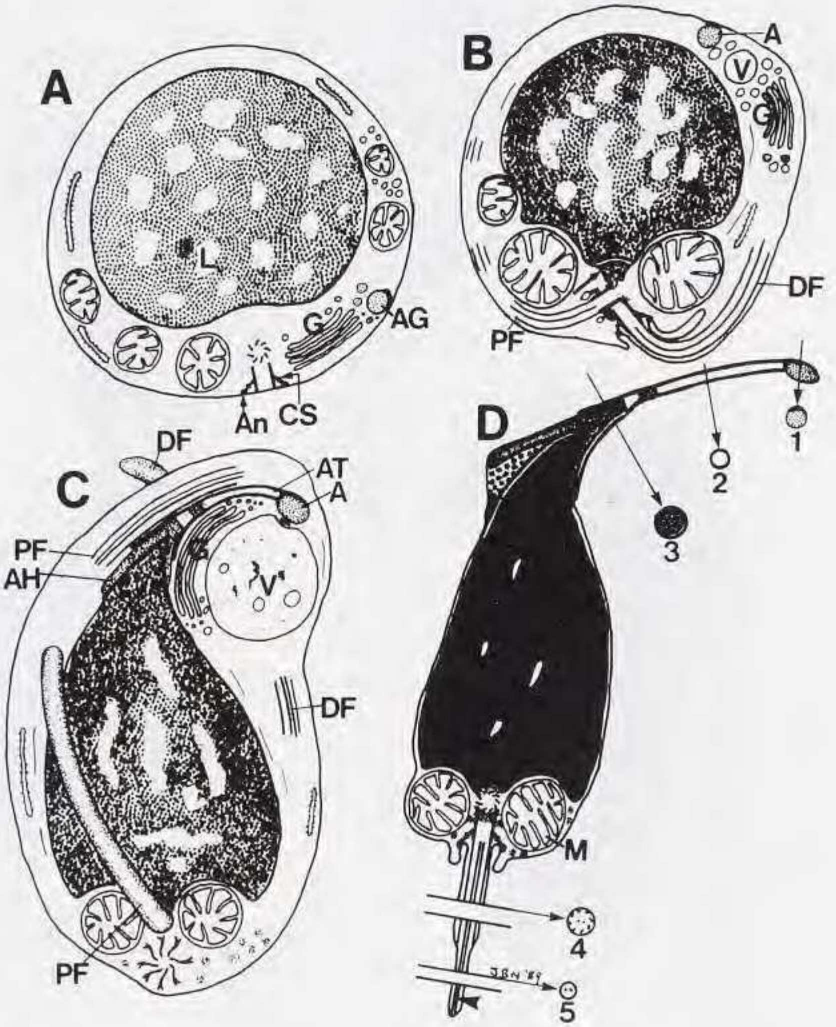


FIG. 39. — Spermiogenesis in *Chaetoderma argenteum*. (redrawn from [18]). **A**: Earliest part of stage A, showing distal centriole attached to plasma membrane by annulus (An) via centriolar satellite complex (CS) with nearby Golgi body (G) producing pro-acrosomal granule (AG). **B**: Proximal (PF) and distal flagella (DF) are visible. Mitochondria are grouped at base of nucleus; Golgi body (G) and proacrosome (A) have migrated anteriorly. Numerous secretory vesicles are fusing to form one larger vesicle (V). **C**: Proximal flagellum (PF) is aligned with apical tube (AT). The acrosome (A) at end of apical tube is connected to large vesicle (V) by fibro-granular material. Golgi body (G) is visible in an apical indentation of nucleus, adjacent to developing apical horn (AH) and apical tube. Distal flagellum exits anteriorly but will eventually encircle spermatid. **D**: Late spermatid showing homogeneously condensed nucleus with few lacunae. Spherical mitochondria (M) surround centrioles. Glycogen granules are visible near apical horn but are also sequestered in mid-piece. Cross sections 1-5: (1) acrosome; (2) apical tube; (3) apical horn; (4) flagellum; (5) end-piece.

*Sperm-egg interaction in Chaetodermomorpha*: The egg of *Chaetoderma*, regrettably, has not been observed with the scanning electron microscope. The only two ripe eggs observed with light microscopy, appeared to have a distinct hull with tiny bumps and small spinous projections (Figs 32, 33). However, at the moment there is no way to be certain that this resembles the hull of chitons, or is formed in a similar way. The mechanics of fertilization may be similar to chitons in that the acrosomal vesicle may digest a pore through the egg envelopes, followed by the apical tube penetrating down to the egg membrane. In both cases the perforatorium that characterizes other molluscs, and Metazoa in general, has been replaced by an anterior projection. In chitons this is a finite extension of the nucleus but in *Chaetoderma* the equivalent projection, the apical tube, is extensible. In response to egg water the apical tube elongated up to 1.5 times its original length, suggesting a novel polymerization process (Fig. 35). T.E.M. sections have not yet revealed what is changing in the anterior structures during elongation, but one suspects the polymerization of actin, which is almost universal among the eukaryotes. Elucidation of the details of sperm-egg interaction in *Chaetoderma* will have to await that elusive but opportune moment.

### *Phylogenetic analysis of Aculifera*

Phylogenetic analysis (Tables 2-4) in MacClade, as well as "branch and bound" analysis in PAUP of 25 morphological characters (including new sperm/egg characters), produced a single minimum length tree (tree length = 67). The tree showed support for classification of three suborders of chitons, in two orders: Lepidopleurina in Lepidopleurida; Chitonina and Acanthochitonina in Chitonida. The Chitonina formed a clade based on synapomorphies for spinous hull projections that are narrow-based, posterior extension of midpiece altering sperm shape, as well as production of long coarse fibres in chromatin condensation. Of the families included in this study, Chitonina included: Callochitonidae, Chaetopleuridae, Acanthopleuridae, Chitonidae, Callistoplacidae, and Ischnochitonidae. Acanthochitonina are separable as a more recent clade by synapomorphies for mitochondrial arrangement in sperm, a fibrous complex in the sperm flagellum, and overall sperm shape, as well as adanal gills and broadly based hull cupules that are not spinous. Acanthochitonina included the families; Lepidochitonidae, Tonicellidae, Mopaliidae, and Acanthochitonidae. The Neomeniomorpha were used for outgroup comparison. The related Chaetodermomorpha clearly aligned with the neomenioids, in spite of different sperm types, based on synapomorphies for spiculate cuticle, distichous radula and other characters discussed below.

## DISCUSSION

### *Phylogenetic relationships among Aculifera*

Phylogeny of Aculifera currently is of great interest to malacologists because, although this group has generally been given a primitive status, recent morphological and molecular analyses place neomenioid aplacophorans basal to all extant Mollusca [67, 69].

Aplacophora (Neomeniomorpha and Chaetodermomorpha) are readily distinguished from Polyplacophora by the following synapomorphies: vermiform body with spiculate cuticle, distichous radula, reduced foot, gonads that open into the pericardium, and a cloaca-like posterior mantle cavity [66, 67, 69]. Also Chaetodermomorpha may be distinguished from Neomeniomorpha based on apomorphies for ectaquasperm structure, lack of seminal receptacles, presence of gills, as well as other characters [reviews: 66, 67, 69].

TABLE 2. — Data matrix and reconstructed states for internal nodes (Note: * = outgroup)

| | 1111111111222222 |
|---|---|
| Node | 1234567890123456789012345 |
| Lepidopleuridae | 0011011420010021211 0?11?? |
| Chaetodermomorpha | 121302141004?0201000000?? |
| Callochitonidae | 2312111411021121311111133 |
| Chaetopleuridae | 2322111111031121412111122 |
| Acanthopleuridae | 2322111111032111412211354 |
| Callistoplacidae | 232211111?32111412111222 |
| Chitonidae | 232211111?32111412211254 |
| Ischnochitonidae | 232211111?32111412111143 |
| Lepidochitonidae | 3340111211123231512121111 |
| Tonicellidae | 2340111212024231512121111 |
| Mopaliidae | 2340111212024231513121111 |
| Acanthochitonida | 234011121 1?23231511331111 |
| Neomeniomorpha* | 0000000000000000000000000 |
| 1 | 0010001410000002000000000 |
| 2 | 0010011410010021211011111 |
| 3 | 2310111411021121311111111 |
| 4 | 2312111411021121311111122 |
| 5 | 2322111111031121412111122 |
| 6 | 2322111111032111412111122 |
| 7 | 2322111111032111412111222 |
| 8 | 2322111111032111412211254 |
| 9 | 2340111211023231511121111 |
| 10 | 2340111211023231512121111 |
| 11 | 2340111212024231512121111 |

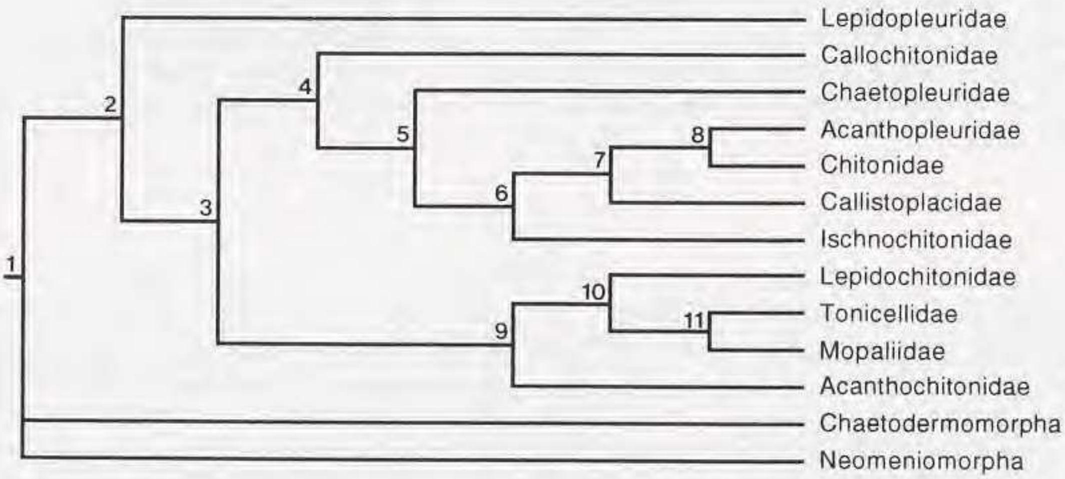


FIG. 40. — Single most parsimonious tree resulting from branch and bound analysis in PAUP of the data matrix in Table 2. Branch and bound search settings were, initial upper bound: unknown (compute via stepwise); addition sequence: furthest; no multistate taxa; unrooted trees rooted using outgroup method. Character state optimization: accelerated transformation (ACCTRAN); tree length = 67; consistency index = 0.970; homoplasy index = 0.030; retention index = 0.967; HI excluding uninformative characters = 0.033; rescaled consistency index = 0.939. The ingroup (1) and all interior nodes (2-11) are labelled. The apomorphy hypotheses supporting each interior node are shown in Table 3.

TABLE 3. — Apomorphy hypotheses for internal nodes of cladogram of Aculiferan phylogeny (Fig. 40). Unless otherwise stated character changes are from 0→1.

| | |
|---|---|
| Node 1→2: | 6,12,16,17(0→2),18,19,21,22,23,24,25 |
| Node 2→3: | 1(0→2),2(0→3),5,10,12(1→2),13,14,17(2→3),20 |
| Node 3→4: | 4(0→2),24(1→2),25(1→2) |
| Node 4→5: | 3(1→2),8(4→1),12(2→3),17(3→4),19(1→2) |
| Node 5→6: | 13(1→2),15(2→1) |
| Node 6→7: | 23(1→2) |
| Node 7→8: | 20(1→2),24(2→5),25(2→4) |
| Node 3→9: | 3(1→4),8(4→2),13(1→3),14(1→2),15(2→3),17(3→5),21(1→2) |
| Node 9→10: | 19(1→2) |
| Node10→11: | 10(1→2),13(3→4) |

TABLE 4. — Characters used in cladistic analysis of Aculifera with character state codes. See Table 2 for data matrix.

Sperm and Egg Data

(1). Acrosome: 0: cone with subacrosomal plate (SA.P.); 1: simple vesicle with SA.P.; 2: complex vesicle with SA.P; 3: simple vesicle with SA.P.

(2). SubAcrosomal Granule: 0: loose granular; 1: rod granular; 2: apical horn & tube; 3: absent

(3). Mitochondrial Associations: 0: mitochondria (M) fused unspiralled; 1: M in ring around central proximal and distal centrioles (PC/DC); 2: offset PC/DC with basal M; 3: offset PC/DC associated with basal not lateral M; 4: offset PC/DC associated with basal & lateral M.

(4). Chromatin Condensation: 0: develop fine long fibres; 1: develop fine then coarse short fibres; 2: develop fine then coarse long fibres; 3: "granular" chromatin.

(5). Golgi Body Production of Acrosome: 0: proacrosomal granule migrates on plasma membrane; 1: proacrosomal vesicles migrate, fuse; 2: proacrosomal granule migrates with Golgi body.

(6). Glycogen Reserves: 0: stored in tail; 1: stored in midpiece; 2: stored in anterior & midpiece.

(7). Flagellar Canal: 0: present but temporary, annulus breaks from D.C.; 1: absent.

(8). Flagellum Reinforced: 0: thickening around axoneme; 1: unilateral thickening of plasma membrane; 2: unilateral fibrous body; 3: absent.

(9). Flagellum Termination: 0: 2 central microtubules elongate+density; 1: 2 centrals, short; 2: 9+2 configuration.

(10). Fertilization Site: 0: anywhere on egg; 1: between hull projections; 2: inside hull projections.

(11). Hull Pores: 0: absent; 1: present

(12). Sperm Head Shape: 0: filamentous; 1: oval ; 2: oval w. nuclear filament; 3: oval w. offset nuclear filament; 4: oval w. apical tube.

(13). Hull Type: 0: smooth; 1: complex spines; 2: simple spines w. modified tips; 3: closed cupules; 4: open cupules.

(14). Cupule Base Size: 0: absent; 1: 5-30 µm; 2: 50-90 µm [71]

(15). Mitochondria: 0: 1-2; 1: 2-4; 2: 4-6; 3: 6-8.

Shell Valve [28,78] and Gill Placement Data [71]

(16). External Covering: 0: spiculate cuticle; 1: 8 shell valves; 2: smooth.

(17). Gills: 0: absent; 1: 2 ctenidia posterior; 2: Type 1 *ad*anal; 3: Type 2 *ad*anal; 4: Type 3 *ad*anal; 5: Type 4 *ab*anal.

(18). Articulementum: 0: absent; 1: present.

(19). Chiton Valve Morphology: 0: absent; 1: modern valve (V) shape; 2: multifissurated terminal Vs 3: post. V. w. post. median sinus; 4: girdle & post.V. fissured.

(20). Insertion Plates: 0: absent; 1: slitted; 2: pectinated; 3: reduced slits.

(21). Girdle Ornamentation: 0: absent; 1: girdle elements similar; 2: potential bristles; 3: girdle fissured; 4: sutural tufts.

(22). Radula Morphology: 0: distichous; 1: docoglossan.

(23). Photoreceptors: 0: absent; 1: macro & micro aesthetes; 2: intrapigmental aesthetes; 3: ocelli.

(24). Gills From Nephropore: 0: absent; 1: 1-2 gills; 2: 3-4; 3: 5-6; 4: 7-8; 5: >9

(25). Gills From Gonopore: 0: absent; 1: 1-3 gills; 2: 4-6; 3: 7-9; 4: >10.

Egg hull projections and associated follicle cells are synapomorphic to chitons, uniting the entire group. Furthermore, all chitons more recent than lepidopleurids are united by having an elongate sperm nuclear filament with reduced acrosome, cupular hull projections, slitted insertion plates and modern gill placement. This evidence supports the theory of THIELE [75] that chitons are a monophyletic group derived from a lepidopleurid-like ancestor, and argues strongly against a polyphyletic origin as proposed by ASHBY [5]. Historically, Polyplacophora have been difficult to resolve because potentially useful characters such as radula morphology, which have an extensive fossil record, are virtually unchanged in the chitons [72]. Other characters, such as girdle morphology or hull spine tip structure, are highly variable and may have arisen independently, or may be polymorphic for a single taxon [28, 71]. EERNISSE [28] first suggested using placement of gills, sperm morphology and egg hull data as a way to develop independent character sets to test the validity of previous phylogenies based on shell valve morphology alone [reviews: 72, 78].

The addition of a new character set based on sperm morphology and mode of fertilization, presented here for the first time, has enabled a preliminary reassessment of chiton phylogeny. The present analysis combined sperm and egg hull data with gill placement and morphology [71], as well as shell valve morphology [28, 78]. The single minimum length tree showed strong support for retaining three suborders of chitons, Lepidopleurina, Chitonina, and Acanthochitonina [71]. Acanthochitonina are more derived than Chitonina based on the sperm fibrous complex, adanal gills and broadly based hull projections. Lepidochitonids clearly are included in Acanthochitonina by possessing each of these synapomorphic characters. Previous classifications based largely on shell valve morphology have given lepidochitonids a rather primitive status placing them between lepidopleurids and other chitonids [reviews: 72, 78]. The present analysis reinstates lepidochitonids in their own family, Lepidochitonidae, as they clearly fall outside the Tonicellidae (based on apomorphies for hull pores, simplified acrosome, and cone-like hulls). This disagrees with a recent classification that placed lepidochitonids within Tonicellidae, but supports the inclusion of both Lepidochitonidae and Mopaliidae within the superfamily Tonicelloidea [71].

The Chitonina have been more difficult to resolve. However, the present analysis provides support for maintaining the suborder Chitonina, as suggested by SIRENKO [71], rather than Ischnochitonina [72, 78]. The superfamily grouping Ischnochitonoidea Dall, 1889 [71], in this analysis included the families Chaetopleuridae, Acanthopleuridae, Chitonidae, Callistoplacidae, and Ischnochitonidae, but excluded the Callochitonidae. Relaxing parsimony by one tree length (= 68) creates an even more paraphyletic assemblage and underscores the need for more synapomorphic characters, particularly for this suborder. More detailed analyses of oogenesis and spermiogenesis (such as acrosome substructure and hull deposition) may provide additional characters that will enable better resolution of this complex taxon.

### *Phylogenetic relationship between Aculifera and other Bilateria: quest for the origin of internal fertilization*

FRANZÉN's hypothesis that the biology of fertilization correlates with sperm morphology works well in most situations [33, 34]. Furthermore, the idea that the "primitive sperm" [33] or "plesiosperm" [45], (which is found throughout the Radiata), represents the primitive condition in basal Metazoa, is not questioned by most biologists. This is partly because sperm of more recent groups usually pass through a stage in spermiogenesis that resembles the plesiosperm type, but also because recent molecular and morphological analyses indicate a monophyletic origin of Metazoa from a diploblastic ancestor [30, 50]. However, the idea that ectaquasperm generally represent the primitive condition wherever they occur in more recent groups [8, 9, 33, 34, 44], is actively debated [22, 23, 38, 46, 47, 54, 60]. For example, JAMIESON [46, page 2] discussed this topic in his recent book on fish evolution: "The term plesiosperm recognizes that, whether or not aquasperm have evolved in some sections of the Metazoa from more complex sperm, the "primitive" sperm facies may be genuinely plesiomorphic in many metazoan groups. This is not to exclude from further investigation the possibility (a) that the earliest metazoans may have been free-spawning with sperm of an even simpler form than the plesiosperm or (b) that they were internally fertilizing with more or less complex sperm".

It was once said that "it is unlikely that primitive spermatozoa from different animal phyla should have evolved independently on several occasions obtaining the same morphology by convergence."[9]. How much more unlikely is it that primitive members of at least twenty animal phyla have evolved a basically similar, infinitely more complex introsperm by convergence? It would seem more logical to assume secondary evolution of the simpler aquasperm from the more complex introsperm. Recent molecular [62] and morphological [67, 69] analyses of neomenioids place them basal to all extant Mollusca. It was surprising to find how typical is the introsperm of neomenioids [22, 23]. It is similar in basic structure and arrangement of organelles to introsperm of primitive members of most bilaterian phyla, including some nemertines [2], bryozoans and entoprocts [35], arthropods [3], annelids [53] as well as some deuterostomes [review: 46]. In particular, neomenioid introsperm closely resemble parasperm of protodrilids [53], a primitive annelid group, formerly included in the now abandoned Archiannelida. The above evidence provides support for current theories that recognize Eutrochozoa (animals with trochophore larvae) as a distinct clade [30, 37, 50], which includes nemertines [76] and perhaps some other invertebrate taxa. Besides the striking similarity to protodrilid parasperm, introsperm of neomenioids have much in common with introsperm of Neritimorpha [15] and "primitive" Caenogastropoda [13, 40], suggesting that current ideas on gastropod evolution [39] also may require revision. Perhaps the Patellogastropoda, presently considered basal to Gastropoda [39], diverged early from a neritimorph-like ancestor?

All Platyhelminthes have introsperm but until the key paper on the primitive turbellarian *Nemertoderma* [77] demonstrated the typical introsperm pattern with a plausible acrosome, rodlike nucleus, elongate midpiece, and single typical flagellum, it had been easy for scientists to dismiss the sperm of Platyhelminthes as highly derived, which most of them are [41]. Since then it has become obvious that introsperm are basal at least to the Platyhelminthomorpha (Platyhelminthes and Gnathostomulida) [6, 73]. Moreover, the above evidence strongly suggests that introsperm are basal to all Bilateria [22, 23]. If true, this would unite Eubilateria and Platyhelminthomorpha as a monophyletic clade.

The basal Platyhelminth groups (Nemertodermatida, Catenulida, Acoela and Macrostomida) are minute organisms, attaining only a few millimeters in length and fractions of a millimeter in width [6]. AX suggests that this is because they all possess the plesiomorphic condition of a simple mouth pore, which limits nutrient uptake and hence body size [6]. More importantly it leaves only minute spaces between the endoderm and ectoderm with little room for expansion of gonads. The corresponding small size of basal Gnathostomulida suggests that the bilaterian stem was a microscopic organism [6]. Based on trace fossil evidence the earliest Bilateria are considered to have emerged as minute wormlike organisms in the Proterozoic, between 600 M and 1000 Myrs ago [25, 26]. BOADEN [11, 12] agreed with this and formulated the "Thyobios" hypothesis which suggested that tiny anaerobic Bilateria evolved in shallow water, anoxic sediments during the earliest emergence of the Metazoa. He suggested that as a consequence of their small size they would have fertilized internally [12]. This correlates with geological evidence of low atmospheric oxygen concentrations until the middle Vendian (550 Myrs) [27, 49]. A late Proterozoic increase in atmospheric oxygen coincides with the emergence of macroscopic Metazoa, first evident in the Ediacaran fauna [25, 27, 49]. However, the trace fossil evidence indicates that Bilateria were well established before this time, and key ichnogenera are traceable through all the early biozones [26].

Furthermore, the suggestion that free spawning always reflects primitive status [33, 44] does not withstand close scrutiny [38, 54]. A study of extant marine invertebrates has shown that reproductive biology (including fertilization biology) depends on a series of covariable reproductive traits [54]. When body length is less than 1mm metazoans fertilize internally with introsperm; only larger bodied forms successfully free-spawn ectaquasperm. Much of the reason for this probably relates to fertilization success. Recent studies have shown that among free-spawners, selection favours larger sperm numbers, larger egg numbers, as well as larger egg size [51, 52]. Other key factors contributing to success include distance between mates, and spawning synchronicity [7, 10, 51, 57]. Tiny triploblastic organisms would not be able to expand the gonads to produce viable numbers of sperm and eggs for free-spawning and selection would favour some form of internal fertilization [54], or hermaphroditism [36].

If it is true that introsperm were basal to Bilateria, then ectaquasperm must have been secondarily developed many times. How could this have occurred? As mentioned, sperm biologists are well aware that all sperm pass through a stage in their ontogeny that resembles the plesiosperm [33, review:47]. Perhaps the re-expression of the plesiosperm form has occurred by truncation of spermiogenesis, a type of progenesis? This has been advanced to explain the presence of plesiomorphic structures in the sperm of schistosomes [48], and probably explains secondary development of ectaquasperm in teleosts [47], as well as sabellid polychaetes [60]. If progenetic spermiogenesis has occurred frequently among animal phyla, we might expect a relatively simple mechanism to control it, perhaps a molecular switch. In vertebrates completion of spermiogenesis is dependent on Sertoli cells [1]. Recently, a regulatory protein was isolated from the round spermatid stage of rats (≈ plesiosperm stage), which was capable of turning on the production of Sertoli cell total protein and transferrin secretion [55]. If these proteins are found to play key roles in the completion of spermiogenesis, the regulatory protein produced by the spermatids could be the kind of molecular switch we are looking for. In the absence of such a regulatory protein, spermiogenesis could be truncated at the plesiosperm stage.

If secondary expression of ectaquasperm has occurred frequently, as suggested, there must be a selective pressure favouring this type of sperm in external fertilization. Evidence indicates that introsperm of some marine invertebrates are capable of swimming effectively in sea water [14,60]. This suggests that the selective advantage to progenetic spermiogenesis may relate more to lower energetic costs and shorter generation times in producing the less complex ectaquasperm, rather than to more efficient locomotion in a sea water medium.

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## REFERENCES

1. ABÉ, S. I., 1987. — In vitro meiosis and early, mid-spermiogenesis in culture of dissociated primary spermatocytes from amphibians. *In:* H. MOHRI, *New Horizons in Sperm Cell Research*. Tokyo, Japanese Science Society Press: 47-53.
2. AFZELIUS, B. A., 1971. — The spermatozoon of the nemertine *Malacobdella grossa*. *Journal of Submicroscopic Cytology*, **3**: 181-192.
3. ALBERTI, G., 1983. — Fine structure of scorpion spermatozoa (*Buthus occitanus*; Buthidae, Scorpiones). *Journal of Morphology*, **177**: 205-212.
4. AL-HAJJ, H. A., 1987. — Ultrastructural study of spermiogenesis in the chiton *Acanthopleura haddoni* from the Gulf of Aqaba (Red Sea). *International Journal of Invertebrate Reproduction and Development*, **12**: 295-308.
5. ASHBY, E., 1931. — Monograph of the South African Polyplacophora (Chitons). *South African Museum, Cape Town, Annals*, **30**: 1-59

6. AX, P., 1985. — The position of the Gnathostomulida and Platyhelminthes in the phylogenetic system of the Bilateria. *In:* S. CONWAY MORRIS, J. D. GEORGE, R. GIBSON & H. M. PLATT, *The Origins and Relationships of Lower Invertebrates*. Oxford, Clarendon Press: 168-180.

7. BABCOCK, R. C., MUNDY , C. N.& WHITEHEAD, D., 1994. — Sperm diffusion models and *in situ* confirmation of long-distance fertilization in the free-spawning asteroid *Acanthaster planci*. *Biological Bulletin* **186**: 17-28.

8. BACCETTI, B., 1985. — Evolution of the sperm cell. *In:* C. B. METZ & A. MONROY, *Biology of Fertilization: Volume 2*. London, Academic Press: 3-58.

9. BACCETTI, B., & AFZELIUS, B. A., 1976. — *The Biology of the Sperm Cell*. Basel, S. Karger: 5-22.

10. BENZIE, J. A. H., & DIXON, P., 1994. — The effects of sperm concentration, sperm:egg ratio, and gamete age on fertilization success in crown-of-thorns starfish (*Acanthaster planci*) in the laboratory. *Biological Bulletin* **186**: 139-152.

11. BOADEN, P. J. S. 1975. — Anaerobiosis, meiofauna and early metazoan evolution. *Zoologica Scripta*, **4**: 21-24.

12. BOADEN, P. J. S., 1977. — Thiobiotic facts and fancies (aspects of the distribution and evolution of anaerobic meiofauna). *Mikrofauna Meeresboden*, **61**: 45-63.

13. BUCKLAND-NICKS, J. A., 1973. — The fine structure of the spermatozoan of *Littorina* (Gastropoda: Prosobranchia), with special reference to sperm motility. *Zeitschrift für Zellforschung*, **144**: 11-29.

14. BUCKLAND-NICKS, J. A. & CHIA, F.-S. 1981. — Locomotion of the filiform sperm of *Littorina* (Gastropoda, Prosobranchia). *Cell and Tissue Research*, **219**: 27-39.

15. BUCKLAND-NICKS, J. A. & CHIA, F.-S., 1986. — Formation of the acrosome and basal body during spermiogenesis in a marine snail, *Nerita picea* (Mollusca: Archaeogastropoda). *Gamete Research*, **15**: 13-23.

16. BUCKLAND-NICKS, J. A., KOSS, R. & CHIA, F.-S., 1988a. — The elusive acrosome of chiton sperm. *International Journal of Invertebrate Reproduction and Development*, **13**: 193-198.

17. BUCKLAND-NICKS, J. A., KOSS, R. & CHIA, F.-S., 1988b. — Fertilization in a chiton: acrosome-mediated sperm-egg fusion. *Gamete Research*, **21**: 199-212.

18. BUCKLAND-NICKS, J. A. & CHIA, F.-S., 1989. — Spermiogenesis in *Chaetoderma* sp. (Aplacophora). *Journal of Experimental Zoology*, **252**: 308-317.

19. BUCKLAND-NICKS, J. A., CHIA, F.-S. & KOSS, R., 1990. — Spermiogenesis in Polyplacophora, with special reference to acrosome formation (Mollusca). *Zoomorphology*, **109**: 179-188.

20. BUCKLAND-NICKS, J. & EERNISSE, D. J., 1992. — Ultrastructure of mature sperm and eggs of the brooding hermaphroditic chiton, *Lepidochitona fernaldi*, Eernisse 1986, with special reference to the mechanism of fertilization. *Journal of Experimental Zoology*, **265**: 567-574.

21. BUCKLAND-NICKS, J., 1993. — Hull cupules of chiton eggs: parachute structures and sperm focusing devices? *Biological Bulletin*, **184**: 269-276.

22. BUCKLAND-NICKS, J. A., 1994 — On sperm ultrastructure and fertilization biology in Aculifera (Mollusca). *Seventh International Symposium on Spermatology, Cairns:* 5.5-5.6.

23. BUCKLAND-NICKS, J. A. & SCHELTEMA, A., 1995. — Was internal fertilization an innovation of early Bilateria? Evidence from the sperm structure of a mollusc. *Proceedings of the Royal Society of London*, series B, **261**: 11-18.

24. BUCKLAND-NICKS, J. A. & HODGSON A. N., 1995. — A re-examination of spermiogenesis in Polyplacophora. *(In preparation).*

25. CONWAY-MORRIS, S., 1993. — The fossil record and the early evolution of the Metazoa. *Nature, London* **361**: 219-225

26. CRIMES, T. P., 1992. — Changes in the trace fossil biota across the Proterozoic-Phanerozoic boundary. *Journal of the Geological Society of London*, **149**: 637-646.

27. DERRY, L. A., KAUFMAN, A. J. & JACOBSEN, S. B., 1992. — Sedimentary cycling and environmental change in the late Proterozoic: evidence from stable and radiogenic isotopes. *Geochemica et Geophysica Acta* **56**: 1317-1329.

28. EERNISSE, D. J., 1984. — Lepidochitona *Gray, 1821 (Mollusca: Polyplacophora), from the pacific coast of the United States: systematics and reproduction*. Ph. D. Thesis, Santa Cruz, U.S.A.

29. EERNISSE, D. J., 1988. — Reproductive patterns in six species of *Lepidochitona* (Mollusca: Polyplacophora). *Malacologia*, **22**: 11-217.

30. EERNISSE, D. J., ALBERT, J. S. & ANDERSON F. E., 1992. — Annelida and Arthropoda are not sister taxa: a phylogenetic analysis of spiralian metazoan morphology. *Systematic Biology*, **41**: 305-330.

31. EERNISSE, D. J. & REYNOLDS P. D., 1994. — Polyplacophora. *In:* F. W. HARRISON, *Microscopic Anatomy of Invertebrates, Volume 5: Mollusca I*. New York, Wiley-Liss: 55-110.

32. FRANZÉN, Å., 1955. — On the spermiogenesis in Solenogastres. *Zoologischer Bidrag från Uppsala*. **30**: 458-491.

33. FRANZÉN, Å., 1956. — On spermiogenesis, morphology of the spermatozoon, and biology of fertilization among invertebrates. *Zoologischer Bidrag från Uppsala* **31**: 355-482.

34. FRANZÉN, Å, 1977. — Sperm structure with regard to fertilization biology and phylogenetics. *Verhandlungen der Deutschen Zoologischen Gesellschaft*, **1977**: 123-138.

35. FRANZÉN, Å, 1981. — Comparative ultrastructural studies of spermatids and spermatozoa in Bryozoa and Entoprocta. *In:* G. P. LARWOOD & C. NIELSEN, *Recent and Fossil Bryozoa*. Denmark, Olsen & Olsen: 83-92.

36. GHISELIN, M. T., 1969. — The evolution of hermaphroditism among animals. *Quarterly Review of Biology*, **44**: 189-208.

37. GHISELIN, M. T., 1988. — The origin of molluscs in the light of molecular evidence. *Oxford Survey of Evolutionary Biology*, **5**: 66-95.

38. HANSON, E. D., 1977. — *The Origin and Early Evolution of Animals*. Middletown, Wesleyan University Press.

39. HASZPRUNAR, G., 1988. — On the origin and evolution of major gastropod groups, with special reference to the Streptoneura. *Journal of Molluscan Studies*, **54**: 367-441.

40. HEALY, J. M., 1982. — An ultrastructural examination of developing and mature euspermatozoa in *Pyrazus ebeninus* (Mollusca, Gastropoda, Potamididae). *Zoomorphology*, **100**: 157-175.

41. HENDELBERG, J., 1977. — Comparative morphology of turbellarian spermatozoa studied by electron microscopy. *Acta Zoologica Fennica*, **154**: 149-162.

42. HODGSON, A. N., J. M. BAXTER, M. G. STURROCK & R. T. F. BERNARD, 1988a — Comparative spermatology of 11 species of Polyplacophora (Mollusca) from the suborders Lepidopleurina, Chitonina and Acanthochitonina. *Proceedings of the Royal Society of London, series B.*, **235**: 161-177.

43. HODGSON, A. N., BAXTER, J. M. & STURROCK, M. G., 1988b — The structure of the chorion of the eggs of some Polyplacophora: correlations with sperm morphology and implications for fertilization. *European Marine Biology Symposium*: 14-15.

44. JÄGERSTEN, G., 1972. — *Evolution of the Metazoan Life Cycle*. New York, Academic Press.

45. JAMIESON, B. G. M., 1987. — A biological classification of sperm types, with reference to annelids and molluscs, and an example of spermiocladistics. *In:* H. MOHRI, *New Horizons in Sperm Cell Research*, Tokyo: Japan Science Society Press: 311-332.

46. JAMIESON, B. G. M., 1991. — *Fish Evolution and Systematics: Evidence from Spermatozoa*. Cambridge, Cambridge University Press: 1-269.

47. JAMIESON, B. G. M. & ROUSE, G. W., 1989. — Spermatozoa of the Polychaeta (Annelida): an ultrastructural review. *Biological Reviews*, **64**: 93-157.

48. JUSTINE, J.-L., 1991. — Phylogeny of parasitic Platyhelminthes: a critical study of synapomorphies proposed on the basis of the ultrastructure of spermiogenesis and spermatozoa. *Canadian Journal of Zoology*, **69**: 1421-1440.

49. KNOLL, A., 1992. — The early evolution of eukaryotes: a geological perspective. *Science*, **256**: 622-627.

50. LAKE, J. A., 1990. — Origin of Metazoa. *Proceedings of the National Academy of Sciences, USA*, **87**: 763-766.

51. LEVITAN, D. R., SEWELL, M. A. & CHIA F. S., 1991. — Kinetics of fertilization in the sea urchin *Strongylocentrotus franciscanus* interaction of gamete dilution, age, and contact time. *Biological Bulletin*, **181**: 371-378.

52. LEVITAN, D. R., 1993. — The importance of sperm limitation to the evolution of egg size in marine invertebrates. *American Naturalist*, **141**: 517-536.

53. NORDHEIM, H. VON, 1989. — Vergleichende Ultrastrukturuntersuchungen der Eu- und Paraspermien von 13 Protodrilus-Arten (Polychaeta, Annelida) und ihre taxonomische und phylogenetische Bedeutung. *Helgoländer Meeresuntersuchungen*. **43**: 113-156.

54. OLIVE, P. J. W., 1985. — Covariability of reproductive traits in marine invertebrates: implications for the phylogeny of the lower invertebrates. *In:* S. CONWAY MORRIS, J. D. GEORGE, R. GIBSON & H. M. PLATT, *The Origins and Relationships of Lower Vertebrates*. Oxford, Clarendon Press. 42-59.

55. ONODA, M. & DJAKIEW, D., 1993. — A 29,000 $M_r$ protein derived from round spermatids regulates Sertoli cell secretion. *Molecular and Cellular Endocrinology*, **93**: 53-61.

56. PEARSE, J. S., 1979. — Polyplacophora. *In:* A. C. GIESE & J. S. PEARSE, *Reproduction of Marine Invertebrates, volume 5; Molluscs: Pelecypods and Lesser Classes*. New York, Academic Press. 27-85.

57. PENNINGTON J. T., 1985. — The ecology of fertilization of echinoid eggs: the consequence of sperm dilution, adult aggregation, and synchronous spawning. *Biological Bulletin*, **169**: 417-430.

58. RICHTER, H-P., 1976. — Feinstrukturelle Untersuchungen zur Oogenese der Käferschnecke *Lepidochitona cinereus* (Mollusca, Polyplacophora). *Helgoländer Meeresuntersuchungen*. **28**: 250-303.

59. RICHTER, H-P., 1986. — Ultrastructure of follicular epithelia in the ovary of *Lepidochitona cinerea* L. (Mollusca: Polyplacophora). *Development Growth and Differentiation* **28**: 42-62.

60. ROUSE, G. W., 1992. — Ultrastructure of sperm and spermathecae in *Micromaldane* spp. (Polychaeta: Capitellidae: Maldanidae). *Marine Biology* **113**: 655-668.

61. ROUSE, G. W. & JAMIESON, B. G. M., 1987. — An ultrastructural study of the spermatozoa of the Polychaetes *Eurythöe companata* (Amphinomidae), *Clymenella* sp. and *Micromaldane* sp. (Micromaldanidae), with definition of sperm types in relation to reproductive biology. *Journal of Submicroscopic Cytology*, **19**: 573-584.

62. RUNNEGAR, B., 1995. — Early evolution of the Mollusca: the fossil record. *In:* J. D. TAYLOR, *Origin and Evolutionary Radiation of the Mollusca.* Oxford, Oxford University Press (in press).

63. RUSSELL-PINTO, F., AZEVEDO, C. & BARANDELA T., 1983. — Fine structure of the spermatozoa of *Chiton marginatus* (Mollusca: Amphineura), with special reference to nucleus maturation. *Gamete Research* **8**: 345-355.

64. RUSSELL-PINTO, F., AZEVEDO, C. & OLIVEIRA E., 1984. — Comparative ultrastructural studies of spermiogenesis and spermatozoa in some species of Polyplacophora (Mollusca). *International Journal of Invertebrate Reproduction and Development*, **7**: 267-277.

65. SAKKER, E. R., 1984. — Sperm morphology, spermatogenesis and spermiogenesis of three species of chitons (Mollusca, Polyplacophora). *Zoomorphology*, **104**: 111-121.

66. SALVINI-PLAWEN, 1985. — Early evolution and the primitive groups. *In:* E. R. TRUEMAN & M. R. CLARKE, *The Mollusca, volume 10, Evolution*. Orlando, Academic Press: 59-150.

67. SCHELTEMA, A. H., 1993. — Aplacophora as progenetic aculiferans and the coelomate origin of mollusks as the sister taxon of Sipuncula. *Biological Bulletin*, **184**: 57-78.

68. SCHELTEMA, A. H., & JEBB, M., 1995. — Natural history of a solenogaster mollusc from Papua New Guinea, *Epimenia australis* (Thiele, 1897) (Aplacophora: Neomeniomorpha). *Journal of Natural History*, **28**: 1297-1318.

69. SCHELTEMA, A. H., TSCHERKASSKY, M. & KUZIRIAN, A. M., 1994. — Aplacophora. *In:* F. W. HARRISON, *Microscopic Anatomy of Invertebrates, volume 5: Mollusca I*, New York, Wiley-Liss: 13-54.

70. SELWOOD (née BEDFORD), L., 1970. — The role of the follicle cells during oogenesis in the chiton *Sypharochiton septentriones* (Ashby) (Polyplacophora, Mollusca). *Zeitschrift für Zellforschung*, **104**: 178-192.

71. SIRENKO, B. I., 1993. — Revision of the system of the order Chitonida (Mollusca: Polyplacophora) on the basis of correlation between the type of gills arrangement and the shape of the chorion processes. *Ruthenica* **3**: 93-117.

72. SMITH, A. G., 1960. — Amphineura. *In:* R. C. MOORE, *Treatise on invertebrate Paleontology, part I, Mollusca 1.* Geological Society of America. New York, University of Kansas Press: 141-176.

73. STERRER, W., MAINITZ, M., RIEGER, R., 1985. — Gnathostomulida: enigmatic as ever. *In:* S. CONWAY MORRIS, J. D. GEORGE, R. GIBSON & H. M. PLATT, *The Origins and Relationships of Lower Invertebrates.* Oxford, Clarendon Press: 181-199.

74. SUMMERS, R. G., 1972. — A new model for the structure of the centriolar satellite complex in spermatozoa. *Journal of Morphology* **137**: 229-242.

75. THIELE, J., 1909. — Revision des Systems der Chitonen. Teil 1. *Zoologica, Bidrag* **22**: 1-70; Teil 2. *Zoologica, Bidrag* **22**: 71-132.

76. TURBEVILLE, J. M., FIELD, K. G. & RAFF, R. A., 1992. — Phylogenetic position of phylum Nemertini, inferred from 18S rRNA sequences: Molecular data as a test of morphological character homology. *Molecular Biology of Evolution* **9**: 235-249.

77. TYLER, S. & RIEGER, R. M., 1975. Uniflagellate spermatozoa in *Nemertoderma* (Turbellaria) and their phylogenetic significance. *Science* **188**: 730-732.

78. VAN BELLE, R. A., 1983. — The systematic classification of the chitons (Mollusca: Polyplacophora). *Informations de la Société Belge de Malacologie.* Série **11**: 1-164.

79. WILEY, E. O., 1981. — *Phylogenetics: the Theory and Practice of Phylogenetic Systematics.* New York, John Wiley & Sons: 1- 439.

80. WILSON, B., VAN DER HORST, G. & CHANNING, A., 1994. — South African anuran sperm head structure, fertilization environment and phylogeny. *7th. International Symposium on Spermatology, Cairns* 1994: 5.44.